Dual Functional Roles for the X-linked Lymphoproliferative Syndrome Gene Product SAP/SH2D1A in Signaling through the Signaling Lymphocyte Activation Molecule (SLAM) Family of Immune Receptors*

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The X-linked lymphoproliferative (XLP) syndrome gene encodes a protein named SAP or SH2D1A that is composed of a single Src homology 2 (SH2) domain. Two models have been proposed for its function in lymphocyte signaling. One postulates that it acts as an inhibitor of interactions between the phosphatase SHP-2 and the immune receptor SLAM. The other suggests that it functions as an adaptor to promote the recruitment of a kinase, FynT, to SLAM. Here, we provide evidence in support of both roles for SAP. Using an array of peptides derived from the SLAM family of receptors, we demonstrate that SAP binds with comparable affinities to the same sites in these receptors as do the SH2 domains of SHP-2 and SHIP, suggesting that these three proteins may compete against one another in binding to a given SLAM family receptor. Furthermore, in vitro and in vivo binding studies indicate that SAP is capable of binding directly to FynT, an interaction mediated by the FynT SH3 domain. In cells, FynT was shown to be indispensable for SLAM tyrosine phosphorylation, which, in turn, was drastically enhanced by SAP. Because SAP also blocked the recruitment of SHP-2 to SLAM in these cells, we propose a dual functional role for SAP in SLAM signaling by acting both as an adaptor for FynT and an inhibitor to SHP-2 binding. The physiological relevance of the dual functional role for SAP is underscored by the observation that disease-causing SAP mutants exhibited significantly reduced affinities to both FynT and SLAM.

The XLP syndrome is an immune deficiency condition marked by an individual’s inability to control acute Epstein-Barr virus infections (1, 2). It manifests itself in various phenotypes, which include fulminant infectious mononucleosis, dysgammaglobulinemia, and malignant B cell lymphomas (1–3). Patients of XLP exhibit specific immune defects such as abnormal natural killer (NK) and T cell-mediated cytotoxicity (4–7). However, the basis for such defects is not yet fully understood.

Cloning of the gene SH2D1A, whose mutation or deletion is responsible for the onset of the XLP syndrome (8–10), and subsequent studies on its encoded protein, termed SH2D1A or SAP, have shed much light on the pathogenesis of the disease. SAP is a protein of 128 amino acids consisting of an N-terminal SH2 domain and a 26-residue C-terminal tail. Its small size has led to the assumption that it may act as a natural inhibitor of SH2 domain-dependent interactions (3, 8). Evidence in support of such a notion comes from the observation that SAP interacts physically with the signaling lymphocyte activation molecule, SLAM, through a Tyr-containing motif that is conserved among members of the SLAM family of lymphocyte surface receptors (3) and that this interaction serves to block a competing interaction between SLAM and SHP-2, a phosphotyrosine phosphatase that plays important roles in various cellular signaling events (11, 12). The structural basis for the specific recognition of SLAM by SAP has recently been unraveled by both x-ray crystallographic and NMR methods (13, 14). We and others (13–15) have shown that the SAP SH2 domain binds to a Tyr-containing motif in SLAM using a unique, “three-pronged” mechanism, rather than a “two-pronged” one employed by most conventional SH2 domains. These studies also identified a consensus motif recognized by the SAP SH2 domain represented by the sequence (Thr/Ser)-X-Tyr(P)-X-X-(Val/Ile), where X denotes any amino acid. The “three-prongs” in the motif correspond to residue Thr or Ser at the N terminus, Tyr(P) in the middle, and Val or Ile at the C terminus (14). Significantly, the presence of any two of the three prongs in a given ligand was shown to be sufficient for its binding to SAP (14, 15).

Protein complexes analogous to the one between SAP and SLAM have also been documented between SAP and other members of the SLAM family of receptors (also called the CD2 receptor family) (3), which now consists of nine related members, including the recently identified SP2000 and SP2001 (16). These receptors are expressed in various subsets of immune cells, such as T, B, and NK cells, and share a similar overall structure characterized by two extracellular Ig-like domains, a single transmembrane segment, and, with the exception of CD48 and BCM1-L, a short cytoplasmic tail (3). Of note, four members of the family, namely SLAM (also called CD150), 2B4 (or CD244), CD84, and Ly-9 (or CD229), contain sequences in their cytoplasmic regions that conform to the consensus motif for interactions between the phosphatase SHP-2 and the immune receptor SLAM.
recognized by SAP (3, 5, 17, 18, 20). Not surprisingly, these receptors have all been shown to interact with SAP in cells (3, 17). However, SAP binds to SLAM constitutively, whereas it binds to the other three receptors in a phosphorylation-dependent manner (5, 17, 18, 20).

The physiological significance of SAP interaction with the SLAM family of receptors was underscored in findings that disease-causing missense SAP mutants displayed markedly reduced affinities for SLAM, both in vitro and in vivo, and were deficient in blocking the interaction between SHP-2 and SLAM (14, 21). Moreover, the binding of SAP to 2B4 appeared to play a pivotal role in regulating target-killing activities of NK cells (5, 18, 20). Thus, NK cells isolated from XLP patients exhibited defects in 2B4-mediated cytotoxicity due to the absence of an active SAP-2B4 complex (5, 22). These observations suggest that SAP plays important roles in signaling through the SLAM family of receptors and that disruption of one or more of these signaling pathways underlies the complex phenotypes of XLP (17).

Notwithstanding the role of SAP as an inhibitor of SH2 domain-mediated interactions, it was recently shown by Veillette and co-workers (23) that SAP promotes a specific association of the T cell-specific tyrosine kinase, FynT, with SLAM in T cells. In addition, SAP-dependent interaction of FynT with SLAM was found to be critical for the tyrosine phosphorylation of SLAM and for the subsequent recruitment of downstream signaling molecules such as SHIP and p62Dok to the receptor. These findings led to the proposal that SAP functions as an adaptor molecule in SLAM signaling rather than as an inhibitor of SH2 domain interaction (23).

To clarify the role of SAP in signaling through the SLAM family of receptors, we systematically mapped the binding sites in these receptors for a series of SH2 domain-containing signaling proteins that include SAP and its homologue EAT-2, SHP-2, SHP-1, SHIP, and FynT, using peptide arrays synthesized on a functionalized cellulose membrane. The notion of SAP as an adaptor in SLAM signaling was explored by examining a direct physical interaction between SAP and FynT in vitro, and the functional significance of SAP-FynT interaction in SLAM phosphorylation and signaling was investigated in HEK293 cells that were made to stably express SLAM. Our results are consistent with a model in which SAP plays a dual functional role, both as an adaptor to bridge an interaction between FynT and SLAM and as an inhibitor to modulate the association of SH2 domain-containing proteins with phosphorylated SLAM.

**EXPERIMENTAL PROCEDURES**

**Subcloning, Expression, and Purification of SH2 Domains**—DNA sequences encoding the SH2 domains of SHIP, FynT, SHP-1, and SHP-2 were amplified by PCR, subcloned into the pGEX4T2 vector, and confirmed by DNA sequencing. Proteins were expressed in *Escherichia coli* and affinity-purified on glutathione-Sepharose beads (Amersham Biosciences). Bound proteins were eluted using 20 mM glutathione in 50 mM Tris and 100 mM NaCl, pH 8.0. Proteins were concentrated, and buffer was changed to phosphate buffered saline (PBS), pH 7.4, prior to use in binding studies.

**Synthesis of Peptide Arrays on Derivatized Cellulosic Membranes and Binding Studies**—An array of undecamer peptides modeled after Tyr-containing sites in the cytoplasmic domains of the SLAM family of receptors were assembled on a derivatized cellulose membrane using Auto-Spot Robot ASP 222 (Abimed) and standard Fmoc (N-(9-fluorenyl)-methoxycarbonyl) solid phase peptide chemistry. The peptide membrane was then washed three times with PBS-T buffer containing 20 mM Tris-HCl, 140 mM NaCl, and 0.1% (v/v) Triton X-100, pH 7.6, and blocked with 5% bovine serum albumin in PBS-T for one h at room temperature. Approximately 1.0 μg of a GST fusion protein was added directly into the blocking solution and incubated with the peptide membrane at room temperature for an additional hour. The membrane was then washed three times with TBS-T and once with TBS prior to addition of anti-GST antibodies. After incubation for 30 min at room temperature, the cellulose sheet was washed three times with TBS and developed with the ECF Western blotting kit (Amersham Biosciences) following the manufacturer’s protocols and documented using a Fluor-S Multi-Imager (Bio-Rad). For reprobing, the peptide sheet was stripped by treating it sequentially with buffer A, containing 8.0% urea, 1% (w/v) SDS, and 0.5% (v/v) β-mercaptoethanol, and buffer B, containing 10% (v/v) acetic acid and 50% (v/v) ethanol, followed by several washes of deionized water. To avoid possible background artifacts resulting from incomplete stripping, binding studies for each SH2 domain were initially conducted using a fresh strip of peptide array generated under identical conditions.

**Cell Culture, Transfection, and Pull-down Studies**—Human embryonic kidney 293 cells (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum (Sigma), 10 units/ml penicillin, and 10 μg/ml streptomycin. For transient protein expression, cells were grown to 70% confluency and transfected with the expression (pMEs) vectors for FynT or mutants (5 μg) by means of LipofectAMINE (15 μl) (Invitrogen). Deletion mutants of FynT were generated by two consecutive rounds of PCR, and the Y417F mutant was prepared by site-directed mutagenesis. A Myc tag sequence was inserted at the C terminus of the SH2 domain of the SH2 domain deletion mutants by PCR to facilitate their detection. Cells were generally harvested between 60–72 h post transfection and lysed in PLC buffer (50 mM Hepes pH7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EDTA, 10 mM Na₃P₂O₇, 100 mM NaF, and 1 mM Na₂VO₄) supplemented with protease inhibitors (1 μM pepstatin A, 2 μM E64, 1 μM bestatin, 10 μM leupeptin, 2 μg/ml aprotinin and 100 μM phenylmethylsulfonyl fluoride (Sigma)).

For pull-down studies, aliquots of cleared HEK 293 lysate containing 50–200 μg of total proteins were mixed with 25 μg of biotin-labeled or GST-fused SAP or SAP mutants in the presence or absence of a SLAM or a control peptide. The generation and production of SAP mutants were reported previously (14). Protein complexes were pelleted using either 30 μl of streptavidin or glutathione-Sepharose beads (Amersham Biosciences), washed five times with PLC buffer, resuspended in 1× SDS loading buffer, and resolved on 10–15% SDS-PAGE. Protein bands were transferred to polyvinylidene difluoride Western blotting membranes (Roche Diagnostics) and incubated in a blocking solution of 5% bovine serum albumin (Sigma) for 1 h at room temperature. The membrane was then washed three times in TBS-T and probed with mouse anti-Fyn monoclonal antibody (Santa Cruz Biotechnology) for 1 h at room temperature. A goat anti-mouse IgG-HRP conjugate (Bio-Rad) was then applied to the membrane for 40 min at room temperature before it was developed using enhanced chemiluminescence (Pierce) on Kodak film according to the manufacturer’s instructions.

**Immunoprecipitation and Western Blot**—The DNA sequence encoding human SLAM was amplified by PCR from an expressed sequence tag clone (Invitrogen), and subcloned into the pRc/CMV2 vector. The pRc/CMV2-SLAM construct was stably transfected in HEK 293 cells by LipofectAMINE followed by G418 restriction. The identity of SLAM was verified by immunoblotting using anti CD150 monoclonal antibodies (Santa Cruz Biotechnology). The HEK 293/SLAM cells transfected with constructs for FynT or FynT mutants and/or SAP were biotinylated using Sulfo-NHS-LC-Biotin (Pierce) and lysed in a buffer containing 1% Nonidet P-40, 150 mM NaCl, 10 mM Tris-HCl, pH 7.7, 1 mM sodium pervanadate, and 10 mM NaF. After precleaving the lysate with preimmune serum absorbed on protein A or G-Sepharose, SLAM was precipitated with SLAM monoclonal antibodies (anti CD150, 4 μg per reaction; lysate containing 800 μg of total protein in 500 μl). The precipitated proteins were separated by SDS-PAGE and transferred under semi-dry conditions to polyvinylidene difluoride membranes (Millipore). Membranes were probed sequentially with anti-Tyr(P) mouse monoclonal antibody (Cell Signaling Tech), rabbit anti-SHP2, mouse anti-SAP (a generous gift from Dr. Cox Terhorst, Harvard) or anti-FLAG monoclonal antibody, anti-Fyn (mouse), or anti-c-Myc (mouse). Biotinylated SLAM was probed with streptavidin-horseradish peroxidase (Sigma). The blotted membranes were developed using enhanced chemiluminescence (Pierce).

**RESULTS**

**Binding of SAP and Related SH2 Domains to Tyr-containing Motifs in the SLAM Family of Receptors**—The cytoplasmic re-
regions of the SLAM family of receptors contain multiple conserved Tyr phosphorylation sites that completely or partially conform to the (Thr/Ser)-X-(Tyr(P))-X-(Val/Ile) motif recognized by the SAP SH2 domain (3, 24–26). To date, four of the SLAM family members, namely SLAM, 2B4, Ly-9, and CD84, have been shown to interact with SAP via one or more of these Tyr phosphorylation sites (5, 17, 18). To determine systematically the preferred binding sites for SAP and related proteins in these receptors, an array of undecamer peptides modeled after these sites was generated on a derivatized cellulose membrane using the SPOT method of multiple peptide synthesis (27). To assess the role of phosphorylation on binding, both phosphorylated and unphosphorylated versions of the same peptides were synthesized. Sequences of the array of peptides and their origins are listed in Table I.

The peptide array was subsequently screened for binding to purified SAP and EAT-2 as well as the SH2 domains of SHIP, SHP-2, SHP-1, and FynT. SHIP and SHP-1 are phosphoinositide and phosphotyrosine phosphatases, respectively, that play important roles in inhibitory signaling through immune receptors (28), whereas the function of SHP-2 in lymphocyte signaling and activation is less well defined. EAT-2 has the same overall structure as SAP and shares extensive sequence identity to the latter (29). Interestingly, EAT-2 is expressed in B cells and macrophages, whereas SAP is mainly found in T and NK cells, suggesting a likely complimentary role for these two homologous proteins in the immune system (3, 29). Because their C-terminal tails are not involved in peptide recognition (14, 29), intact SAP and EAT-2 were used in the binding studies together with the other SH2 domains.

As seen in Fig. 1, with the exception of peptide 10-Tyr(P) (phosphotyrosine-containing peptide number 10) derived from the Tyr-581 site in Ly-9 (see Table I), all phosphorylated (Tyr(P)) peptides in the array displayed strong binding to SAP, implying that SAP can be recruited to each of the four SLAM family members via multiple sites. Interestingly, only two unphosphorylated (Tyr) peptides, namely peptides 3-Tyr (Tyr-containing peptide number 3) from the SLAM Tyr-281 site and 8-Tyr (Tyr-containing peptide number 8) from CD84 Tyr-262, were capable of binding to SAP. This result is consistent with those reported in the literature showing that SAP could associate with SLAM and CD84 in a constitutive manner (8, 17), whereas it could associate with 2B4 and Ly-9 only following their tyrosine phosphorylation (17, 18). In comparison, the homologous protein EAT-2 exhibited an almost identical binding pattern to the array of Tyr(P)-containing peptides as SAP (Fig. 1). However, EAT-2 displayed only weak and, most likely, nonspecific binding to the array of Tyr-containing peptides. It can thus be predicted from this result that EAT-2 can only be recruited to the SLAM receptors in a phosphorylation-dependent manner, as shown in a recent study by Morra et al. (29) to be indeed the case.

The N-terminal SH2 domain of SHP-2 was used because no significant binding was detected for the C-terminal SH2 domain (not shown). All proteins were used as GST-fusion. Bright (fluorescent) spots indicate positive binding. See "Experimental Procedures" for details of experimentation.

**Fig. 1.** Comparable binding patterns for SAP, SHP2, and SHIP to SLAM receptor family members. Binding patterns for SAP, EAT-2, and SHIP domains from SHP-2, SHIP, FynT, and SHP-1 to an array of peptides derived from the SLAM family of receptors (see Table I for sequence information) are shown. The N-terminal SH2 domain of SHP-2 was used because no significant binding was detected for the C-terminal SH2 domain (not shown). All proteins were used as GST-fusion. Bright (fluorescent) spots indicate positive binding. See “Experimental Procedures” for details of experimentation.

| Spot No. | Amino acid sequence | Origin | Ref. |
|----------|---------------------|--------|------|
| 1        | Pro-Cys-Thr-Ile-Tyr(P)-Val-Ala-Ala-Thr-Glu | SLAM Tyr-307 | 8    |
| 2        | Asn-Ser-Ile-Thr-Val-Tyr(P)-Ala-Ser-Val-Thr-Leu | SLAM Tyr-327 | 8    |
| 3        | Lys-Ser-Leu-Thr-Ile-Thr(P)-Ala-Gln-Val-Gln-Lys | SLAM Tyr-281 | 8    |
| 4        | Glu-Phc-Leu-Thr-Ile-Tyr(P)-Glu-Asp-Val-lys-Asp | 2B4 Tyr-286 | 24   |
| 5        | Gly-Gly-Ser-Thr-Ile-Tyr(P)-Ser-Met-Ile-Gln-Ser | 2B4 Tyr-294 | 24   |
| 6        | Pro-Ala-Tyr-Thr-Leu-Tyr(P)-Ser-Leu-Ile-Gln-Pro | 2B4 Tyr-312 | 24   |
| 7        | Phe-Asn-Ser-Thr-Ile-Tyr(P)-Glu-Val-Ile-Gly-His | 2B4 Tyr-337 | 24   |
| 8        | Ser-Lys-Lys-Thr-Ile-Tyr(P)-Thr-Ile-Met-Ala | CD84 Tyr-262 | 25   |
| 9        | Pro-Val-Asn-Thr-Val-Tyr(P)-Ser-Glu-Val-Gln-Phe | CD84 Tyr-299 | 25   |
| 10       | Glu-Gly-Gln-Ala-Asp-Tyr(P)-Asp-Pro-Val-Thr-Pro | Ly-9 Tyr-551 | 26   |
| 11       | Asp-Pro-Val-Thr-Pro-Tyr(P)-Val-Thr-Glu-Val-Glu | Ly-9 Tyr-588 | 26   |
| 12       | Gly-Glu-Asn-Thr-Val-Tyr(P)-Ala-Gln-Val-Phe-Asn | Ly-9 Tyr-602 | 26   |
levels of fluorescence polarization, to yield the corresponding Kd protein concentrations (X) from a single measurement. They were fitted to the equation Y and FynT to fluorescein-labeled SLAM Tyr(P)-281 peptide. Data points shown represent relative fluorescence polarization values (Y) at various due to weak binding.

The finding that FynT associates with SLAM in a SH2 domain alone. This was likely due to the fact that the SH2 domains displayed a higher affinity than the N-terminal anchoring sequence containing KGG to avoid end effects. See legend to Fig. 2 for the derivation of Kd values. ND, not determined due to weak binding.

### Table II
Relative affinities of various SH2 domains for fluorescein-labeled peptides SLAM Tyr(P)-281 and Tyr-281

Sequences of the SLAM Tyr(P)-281 and Tyr-281 peptides correspond to those of spots 3-Tyr(P) and 3-Tyr in the peptide array (see also Table I). The peptides were labeled with fluorescein using an N-terminal anchoring sequence containing KGG to avoid end effects.

| SH2 protein | SLAM Tyr(P)-281 Kd (mM) | SLAM Tyr-281 Kd (mM) |
|-------------|--------------------------|----------------------|
| SH2D1A      | 0.23 ± 0.03              | 0.60 ± 0.07          |
| EAT-2       | 0.49 ± 0.10              | > 50                 |
| SHP-2N      | 2.08 ± 0.24              | ND                   |
| SHP-2C      | 19.06 ± 4.11             | ND                   |
| SHP-2NC     | 0.58 ± 0.13              | ND                   |
| SHIP        | 1.34 ± 0.14              | ND                   |
| SHP-1N      | 25.38 ± 1.85             | ND                   |
| SHP-1C      | 25.24 ± 0.75             | ND                   |
| FynT        | 7.48 ± 0.82              | > 50                 |

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displayed a negative to weak binding to both the Tyr(P)- and Tyr-containing peptides. Collectively, these results indicate that SAP, EAT-2, SHP-2 and SHIP may be bona fide ligands for the SLAM family of receptors, whereas SHP-1 and FynT are not.

To quantitate these interactions, we measured the affinities of the above SH2 domains for two peptides derived from the Tyr-281 site in SLAM by fluorescence polarization (15). In agreement with results obtained using peptide arrays, the SH2 domains of SHIP and SHP-2 (N- or N, C-tandem) were found to bind strongly to the SLAM Tyr(P)-281 peptide, with Kd values for the corresponding peptide-protein complexes ranging from sub to low micromolar (Fig. 2, and Table II), which are comparable with those of the SAP- or EAT-2-peptide complexes (Table II). It was also observed that the C-terminal SH2 domain of SHP-2 was essentially inactive in binding and that the tandem SH2 domains displayed a higher affinity than the N-terminal SH2 domain alone. This was likely due to the fact that the former was more stable than the latter (data not shown) rather than because of a cooperative effect of the two SH2 domains in tandem. In contrast, the SH2 domains of FynT and SHP-1 showed significantly reduced binding to peptide SLAM Tyr(P)-281. As seen also in the peptide array studies, none of the SH2 domains, except SAP, exhibited significant binding to the SLAM Tyr-281 peptide.

**SAP Interacts with FynT in Vitro as Well as in Vivo, an Interaction Augmented by Concomitant Binding of SLAM Peptides**—The finding that FynT associates with SLAM in a SAP-dependent manner (21) suggests that SAP may either indirectly or directly interact with SLAM. To explore the latter possibility, we employed a purified SAP protein, either as GST-fusion or labeled with biotin, in an attempt to pull down FynT from the lysate of HEK 293 cells that transiently overexpress FynT. As seen in Fig. 3A, both GST-SAP and biotin-SAP could pull down FynT, whereas GST and glutathione- (lane 1) or streptavidin-Sepharose beads (lane 4) failed to do so, indicating that SAP interacts specifically with FynT in vitro. To find out whether SAP and FynT can form a complex in vivo, we transiently expressed the two proteins in HEK 293

![Figure 2](image1.png)

**Fig. 2. Binding of SH2 domains to a SLAM peptide.** Binding curves for the tandem SH2 domains of SHP-2 and the SH2 domains of SHIP and FynT to fluorescein-labeled SLAM Tyr(P)-281 peptide. Data points shown represent relative fluorescence polarization values (Y) at various protein concentrations (X) from a single measurement. They were fitted to the equation Y = B_{max} \times X/(K_{d} + X), where B_{max} represents maximal levels of fluorescence polarization, to yield the corresponding K_d values using the software Prism 3.0 (Graphpad Software Inc.). Average K_d values from two independent experiments are reported in Table II.

![Figure 3](image2.png)

**Fig. 3. SAP interacts simultaneously with FynT and SLAM.** A, binding of SAP to FynT assayed in a pull-down study using GST-SAP and biotinylated SAP (Bt-SAP). GST, glutathione- (lane 1) and streptavidin-Sepharose beads (lane 4) were included as controls. The same amount of lysate (150 µg of total protein), protein (25 µg), and beads (30 µl) were used for all lanes. Protein bands corresponding to those of FynT were indicated. WCL, whole cell lysate. B, detection of FynT in anti-SAP immunoprecipitates (IP) from lysates of HEK 293 cells either transfected with SAP alone or with both SAP and FynT constructs. The blot was reprobed with anti-SAP antibody to show comparable amounts of SAP precipitated. Lysates were pre-cleared using normal serum prior to IP. WB, Western blot. C, binding of biotinylated SAP to FynT in the absence or presence of 10–100 µM of SLAM Tyr(P)-281 (SLAM-pY281) or SLAM Tyr-281 (SLAM-Y281) peptides (corresponding to peptide 3-Tyr(P) and 3-Tyr in Table I, respectively). Experimental conditions were the same as for panel A. Similar results were obtained using GST-SAP (data not shown). D, binding of biotinylated SAP to FynT in the presence or absence of a control peptide from the Numb-associated protein Nak containing the sequence GFSNMSFEDFP (30).
cells. As shown in Fig. 3B, FynT is detected in anti-SAP immunoprecipitates from cells simultaneously expressing the two proteins but not in cells expressing SAP alone.

Because SAP also binds to SLAM through the Tyr-281 site, we were interested in finding out whether SAP functions as an adaptor for FynT and SLAM or whether the two interactions compete and are thereby mutually exclusive. To this end, we examined the effects of two SLAM peptides, namely SLAM Tyr(P)-281 and SLAM Tyr-281, in SAP-FynT interaction. As shown in Fig. 3C, neither the phosphorylated nor the unphosphorylated SLAM peptide inhibited the interaction of SAP with FynT. On the contrary, these two peptides appeared to enhance SAP binding to FynT. This “enhancing” effect was particularly prominent for the SLAM Tyr-281 peptide, which was found to increase the amount of SAP-associated FynT significantly even when applied at 10 μM. By comparison, an unrelated peptide
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Regulation of SLAM Signaling by the Formation of a FynT-SAP-SLAM Complex—To validate the role of SAP as an adapter for SLAM and FynT in vivo, we derived a human embryonic HEK 293 kidney cell line that stably expresses the SLAM receptor by means of G418 restriction. FynT or a FynT mutant was then transiently expressed in these cells with or without co-expression of SAP. Receptor-associated SAP, FynT (or mutants), and SHP-2 in anti-SLAM immunoprecipitates were assayed respectively using specific antibodies against these proteins. As shown in Fig. 5A, SAP associated with SLAM constitutively in cells transfected with an empty vector (panel ii, lane 4). However, the level of receptor-bound SAP was dramatically increased in cells bearing FynT (Fig. 5A, lane 2), an observation that is in agreement with previous reports (8, 23). In contrast, basal levels of SLAM-associated SAP were detected in cells expressing FynT mutant ΔSH3 or Y417F, suggesting that the SH3 and kinase domains of FynT are important for SAP-SLAM interaction. Interestingly, the FynT ΔSH2 mutant was also largely ineffective in promoting the SAP-SLAM complex, as the amount of the SLAM-bound SAP in cells expressing this mutant was only moderately higher than the basal level (Fig. 5A, panel ii, lane 6). Binding of SAP to SLAM appeared to be correlated with the presence of tyrosine phosphorylation of the receptor. Although SLAM phosphorylation was detectable in the cells containing FynT but not SAP (Fig. 5A, panel iv, lane 1), the presence of SAP drastically increased the extent of SLAM phosphorylation (Fig. 5A, panel iv, lane 2). In comparison, a complete absence or significant decrease in SLAM phosphorylation was observed in cells expressing any of the FynT mutants, regardless of the presence or absence of SAP. The deficiency of the FynT ΔSH2 and ΔSH3 mutants in phosphorylating SLAM cannot simply be attributed to their relatively low levels of expression compared with wild-type FynT (Fig. 5B), but rather their incompetence in SLAM phosphorylation reflects the importance of the SH2 and SH3 domains of FynT, especially the latter, in proper signaling of SLAM.

Elevated levels of SLAM phosphorylation also correlated with the increased binding of FynT to the receptor (Fig. 5A, panels iii and iv, lanes 1 and 2). Although FynT appeared to associate weakly with SLAM in the absence of SAP, possibly mediated by a weak binding of its SH2 domain to phosphorylated SLAM (Fig. 2; note that no SLAM phosphorylation or receptor binding was detected on the FynT kinase-dead mutant Y417F), SAP was seen to significantly enhance FynT binding to the receptor (Fig. 5A, panels ii and iii, lanes 1 and 2). It should be noted that the amount of SLAM-associated FynT ΔSH2 and ΔSH3 mutants were difficult to assess because they comigrated with the immunoglobulin heavy chain. These results demonstrate that SAP plays a role of an adapter in promoting the recruitment of FynT to SLAM. Interestingly, the binding of SAP to SLAM was seen to also block the interaction of SHP-2 with the receptor (Fig. 5A, panel v, lanes 1 and 2). Thus, SAP is capable of not only bridging the interaction of FynT with SLAM but also inhibiting the binding of SHP-2 to phosphorylated SLAM.

Disease-causing SAP Mutants Exhibit Defects in Binding to FynT—A number of missense point mutations have been identified in the SH2D1A gene isolated from XLP patients. These mutations generally result in single amino acid...
changes within the protein’s SH2 domain, except that, in one case, the stop codon is mutated to an Arg, which leads to the addition of 11 extra amino acids to the C terminus of SAP (the “Tail” mutant). We previously reported that a group of ten SAP mutants associated with XLP displayed reduced affinities for SLAM and SLAM-derived peptides and that these mutants were incompetent in blocking the interaction between SHP-2 and SLAM compared with wild-type SAP (14, 21). To assess the relevance of the SAP-FynT interaction to the pathogenesis of the XLP syndrome, we examined the binding of the same group of mutants to FynT in a GST pull-down assay. As shown in Fig. 6, with the exception of the Tail mutant, binding of each of the remaining nine mutants to FynT was either significantly compromised or completely abolished, compared with SAP. It should be noted that the Tail mutant bound to FynT as strongly as wild-type SAP, implying that this mutant is defective in other characteristics than just its ability to interact with FynT.

**DISCUSSION**

A central question regarding the role of SAP in the pathogenesis of the XLP syndrome is how SAP regulates the signaling and, thereby, the activities of immune cells such as the T and NK cells. The small size of the SAP protein favors the notion that it may act as a natural inhibitor to block or modulate the binding of other SH2 domain-mediated interactions. A number of studies suggest that this mechanism may be physiologically relevant because SAP is indeed capable of inhibiting the binding of SHP-2 to SLAM in COS-7 cells and to the homologous receptor 2B4 in NK cells (5, 8). Nonetheless, this mechanism of SAP action seems to contradict the notion of SAP acting as an adaptor in SLAM receptor signaling.

Our observation that the SH2 domains of SHIP and SHP-2 bind to the same sites in the SLAM family of receptors as does SAP strongly suggests that these three proteins are binding partners competing for the same receptors. In the case of SLAM, SAP binds with the highest affinity among the three proteins to the Tyr-281 site, and it therefore should compete favorably against SHP-2 in binding to the same site as shown previously (8, 21) as well as in the present study. However, because all four members of the SLAM family of receptors examined in this study contain multiple tyrosine phosphorylation sites, the efficiency of these SH2 domain-containing proteins in competing against each other would depend on their relative affinities for these sites and their local concentrations.

Because of its small size and single domain structure, the notion of SAP acting as an adaptor in SLAM receptor signaling is not readily comprehended, because adaptor proteins often contain multiple protein-interacting domains and motifs (19). Our finding that SAP simultaneously binds to tyrosine sites in the SLAM receptor and to the SH3 domain of the FynT kinase suggests that it can indeed serve a role as an adaptor for the recruitment of FynT to the receptor. Although the structural basis for such a unique interaction is not yet clear, the functional consequence is that more kinase is recruited to the receptor by SAP, resulting in enhanced receptor phosphorylation. It should be pointed out that basal levels of receptor-associated FynT and receptor phosphorylation are detectable in cells containing no SAP. Although our results agree with those reported by Sayos et al. (8), they differ from those by Veillette and co-workers (23). This discrepancy may be due to higher kinase concentrations in the systems of Sayos et al. (8) and ours as a result of transient overexpression of FynT than those in the latter study. Nonetheless, our observation that a small amount of FynT can be recruited to the receptor independent of SAP may be functionally significant. It has been previously shown, and confirmed in the present study, that SAP interacts with the NK cell-activating receptor 2B4 in a phosphorylation-dependent manner (5, 17, 18, 20). Apparently, a basal level association of a kinase with the receptor would facilitate its initial phosphorylation and hence the subsequent recruitment of SAP.

Based on findings reported here and by others (8, 23), we propose a unified model for SAP signaling through SLAM, where it plays a dual functional role both as an adaptor for FynT and as an inhibitor of SH2 domain-mediated interactions. In this model, the initial interaction takes place between SAP and SLAM, which can occur constitutively. The binding of SAP to SLAM promotes its association with FynT through an adaptor function of SAP. Subsequently, receptor-associated FynT can phosphorylate Tyr residues in the cytoplasmic region of the receptor or of a neighboring receptor molecule following receptor cross-linking or activation. This, in turn, creates docking sites for SH2 domain-containing proteins such as SHP-2 and SHIP. Because SAP is also capable of binding to the same phosphorylated sites, the amount of receptor-associated SHP-2 or SHIP and, thereby, the outcome of downstream signaling, would depend on the relative affinities and abundance of these proteins in the cell at a given time.

A similar mechanism could also be employed in principle by other members of the SLAM family of receptors. CD84 contains a site (Tyr-262) that can bind SAP constitutively. The receptors 2B4 and Ly-9 lack such a site, and their association with SAP could only concur with tyrosine phosphorylation. A basal level phosphorylation of these receptors may contribute to their initial recruitment of SAP and, subsequently, of FynT (or another kinase), ultimately leading to full-fledged phosphorylation of the receptors. Although further studies are needed to substantiate this model, it is of particular interest to find out that disease-causing SAP mutants display defects in binding to both SLAM and FynT.

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