Disease-Causing SAP Mutants Are Defective in Ligand Binding and Protein Folding†

Chengjun Li,‡,§ Cristiana Iosef,‡,§ Christina Y. H. Jia,§ Theofanis Gkourasas,§ Victor K. M. Han,# and Shawn Shun-Cheng Li*,§,#

Department of Biochemistry, Faculty of Medicine and Dentistry, University of Western Ontario, London, Ontario N6A 5C1, and Child Health Research Institute, 800 Commissioners Road East, London, Ontario N6C 2V5, Canada

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ABSTRACT: The X-linked lymphoproliferative (XLP) syndrome is caused by mutations or deletions in the *SH2D1A* gene that encodes an SH2 domain protein named SH2D1A or SAP. The identification of a number of missense mutations within the protein's SH2 domain, each of which can directly cause disease, provides a unique opportunity to investigate the function of an interaction protein module, SH2, in the pathogenesis of XLP. We show here that SAP mutants found in XLP patients are defective in binding its physiological ligands signaling lymphocyte activating molecule (SLAM), a co-receptor in T cell activation, and Fyn, a Src family protein tyrosine kinase. Consequently, these mutants are deficient in signaling through the SLAM receptor. This is reflected by compromised abilities for the mutants to recruit Fyn to SLAM and to activate Fyn, by reduced phosphorylation of the receptor, and by deficiencies for the mutants in blocking binding of SHP-2 to SLAM. Furthermore, all mutants examined are defective in protein folding as manifested by their significantly reduced melting temperatures upon thermal denaturation, compared to that of SAP. Taken together, these results suggest that defects in ligand binding, receptor signaling, and protein folding collectively contribute to the loss of function for disease-causing SAP mutants.

INTRODUCITON

The X-linked lymphoproliferative (XLP)[1] syndrome, or Duncan disease, is a rare immunodeficiency condition affecting only males (*1*−*3*). XLP patients exhibit abnormalities in the functions of T and natural killer (NK) cells, which are incapable of controlling the proliferation of Epstein−Barr virus-infected B cells. This can lead to a variety of clinical symptoms, ranging from infectious mononucleosis to B cell lymphoma (*1*−*3*), and culminates in premature death. The genetic defect of the disease was mapped to a gene variously named *SH2D1A*/*SAP*/*DSHP* (*5*−*7*), which encodes a protein of 128 amino acids (designated SAP herein) containing a single SH2 domain and a 26-residue C-terminal tail. SAP homologues have been described in several other species ranging from cotton-top tamarin to mouse (*8*), suggesting an evolutionarily conserved role for SAP. The normal cellular function of SAP, however, is not fully understood at present. Because SAP is expressed primarily in T and NK cells, it is suggested to play a part in modulating the proliferation and activities of these cells (*9*). SAP is capable of interacting with the signaling lymphocyte activating molecule (SLAM), a co-stimulatory receptor for T cell activation, and with several additional members of the CD2 family of immune receptors, including CD84, Ly-9, and 2B4 that are present on the surfaces of various types of lymphocytes (*4*, *10*−*13*). The SAP−SLAM interaction was shown to block the binding of a downstream phosphotyrosine phosphatase, SHP-2, to SLAM (*6*). SAP may also function as an adaptor in SLAM signaling (*14*−*17*) since it can simultaneously bind to SLAM and Fyn, and thereby, recruit Fyn to the SLAM receptor. It is therefore likely that SAP plays a dual functional role in SLAM signaling by acting, on one hand, as an adaptor for Fyn and SLAM, and on the other hand, as a modulator for binding of downstream molecules to the receptor (*15*).

All functions of SAP identified to date have been ascribed to its SH2 domain, which recognizes a conserved sequence motif [T/S]x(p)Yxx[I/V], where x denotes any amino acid (*18*, *19*). Although SAP can bind its ligands in a phosphorylation-independent manner, phosphorylation of the Tyr residue enhances binding (*18*−*20*). Sequences that conform to the SAP-binding motif are present in the cytoplasmic domains of SLAM and other members of the CD2 family (*4*). Since this motif bears resemblance to the immunoreceptor tyrosine-based inhibitory motif (ITIM), which has the generic sequence [I/V]xYxxL (*21*), it is given an acronym ITSM for immunoreceptor tyrosine-based switching motif (*22*). While the "switching" role for the ITSM sequence awaits clarification, it is interesting to note that binding of

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* To whom correspondence should be addressed. Tel.: 519−850−2910. Fax: 519−661−3175. E-mail: sli@uwo.ca.

‡ These authors contributed equally to this work.

§ University of Western Ontario.

Child Health Research Institute.

[1] Abbreviations: CD, circular dichroism; HEK, human embryonic kidney; GST, glutathione S-transferase; ITIM, immuno-receptor tyrosine-based inhibitory motif; ITSM, immuno-receptor tyrosine-based switching motif; SAP, SLAM-associated protein; SH2, Src homology 2; SHP-2, SH2-containing protein tyrosine phosphatase 2; SLAM, signaling lymphocyte activation molecule; wt, wild type; XLP, X-linked lymphoproliferative syndrome.

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SAP to SLAM or other members of the CD2 family indeed affect their interactions with the SH2-containing inositol phosphatase (SHIP) and SHP-2 (*6*, *10*−*13*, *22*).

The central role of the SAP SH2 domain in the pathogenesis of XLP is underscored by the large number of missense mutations identified within this domain (*22*−*26*). These mutants provide unique tools for dissecting the molecular mechanism of the XLP syndrome. It is particularly interesting that, while some mutations occur at the ligand-binding surfaces of the SAP SH2 domain, which can have a direct impact on ligand recognition, others take place at sites removed from these surfaces (*17*, *19*, *20*). These latter mutations may alter the structure or folding characteristics of SAP such that they affect binding in an indirect manner. We describe here the biochemical and biophysical characterization of a group of six disease-causing, naturally occurring SAP mutants. Binding studies and thermal denaturation analysis indicate that defects in ligand-binding and protein folding collectively contribute to the malfunction of these mutants, and therefore, to the pathogenesis of the XLP syndrome.

EXPERIMANTAL PROCEDURES

Subcloning, Expression, and Purification of SAP and Its Mutants. Human SAP cDNA was amplified by PCR and subcloned into a pGEX 4T2 vector. Disease-causing SAP mutants were reproduced in vitro by Quickchange site-directed mutagenesis (Stratagene, La Jolla, CA) using SAP-pGEX4T2 as template. Proteins were expressed in *Escherichia coli* as GST-fusion and affinity-purified on glutathione sepharose beads (Amersham Pharmacia Biotech.) according to the manufacturer's recommendations. Bound proteins were eluted using 20 mM gluotathione in 50 mM Tris and 100 mM NaCl, pH 8.0, and were concentrated and buffer-exchanged to phosphate buffered saline (PBS), pH 7.4 prior to use in binding studies. For unfolding experiments, the GST fusion proteins were treated with thrombin to generate isolated SAP and mutants, which were further purified on a Superdex G75 column on an AKTA FPLC system (Amersham-Pharmacia Biotech.).

For binding studies involving the Fyn SH3 domain, a cDNA fragment encoding the human FynT (T cell-specific Fyn) was amplified by PCR and subcloned into the pGEX4T2 vector. Isolated Fyn SH3 domain was generated by thrombin cleavage of the GST fusion. For fluorescein labeling (see also the next section), Fyn SH3 protein in 50 mM $NaHCO_3$, 100 mM NaCl, pH 8.5 was treated for 2 h at room temperature with 5-(and-6)-carboxyfluorescein succinimidyl ester (Molecular Probes, Oregon) at 1:1.5 molar ratio. The unreacted fluorescein derivative was separated from the labeled protein by gel-filtration.

Fluorescence Polarization Measurements. Fluorescein labeling of SLAM-(p)Y281 peptides and polarization measurements were as previously described (*18*, *19*). Equilibrium binding isotherms were obtained by titrating the fluorescent peptide or labeled Fyn with purified SAP or a mutant protein (in GST-fusion). Binding curves were generated by fitting data to a nonlinear regression model using Prism (GraphPAD Software, San Diego, CA), which also gave the corresponding dissociation constant (K_d) values. GST alone did not show appreciable binding to any of the fluorescent probes.

Cell Culture, GST Fusion Pull-Down, Co-Immunoprecipitation (co-IP), and Western Blot. Human embryonic kidney 293 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 10 units/mL penicillin, and 10 μg/mL streptomycin. The cells were transiently transfected with a Fyn-expressing vector (pMES) by means of LipofectAMINE (Invitrogen) and harvested in 60 h in a buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM $Na_2P_2O_7$, 10 mM NaF, and 1 mM Na_3VO_4, and supplemented with protease inhibitors (1 μM pepstatin A, 2 μM E64, 1 μM bestatin, 10 μM Leupeptin, 1 μg/mL aprotinin, and 100 μM PMSF, Sigma Co.). For pull-down studies, aliquots of cleared 293 cell lysate containing 100 μg of total proteins were mixed with GST, GST-SAP, or a GST-SAP mutant immobilized on glutathione agarose beads for 2 h at 4 °C. Bound proteins were collected by centrifugation and resolved on SDS−PAGE. Protein bands were blotted onto PVDF membranes (Roche Diagnostic) and detected with mouse anti-Fyn monoclonal antibodies (Santa Cruz Biotech., Inc.).

Human SLAM cDNA was PCR-amplified and subcloned into a pRc/CMV2 vector. The 293 cells that stably express SLAM were obtained by G418 restriction. The identity of SLAM was verified by immunoblotting using mouse anti-CD150 (SLAM) monoclonal antibodies (Santa Cruz Biotech.). For co-IP studies, the 293-SLAM cells were cotransfected with expressing constructs for Fyn and SAP (or a SAP mutant, in pFLAG-CMV2), respectively. After 48 h, the transfectants 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, 10 mM NaF. The lysate was cleared with preimmune serum absorbed on protein A or G Sepharose beads, and subjected to immunoprecipitation using anti-SLAM antibodies. The immunoprecipitates were resolved in SDS−PAGE and transferred to a PVDF membrane, which was then probed sequentially with antibodies against SLAM, pTyr (Cell Signaling Tech.), SAP (a gift from Dr. Rusung Tan, University of British Columbia), Fyn, and SHP-2. Reciprocal Co−IP experiments were carried out using anti-FLAG (SAP) or anti-Fyn, and the corresponding blots probed with appropriate antibodies.

CD Measurements and Thermal Denaturation. CD spectra were recorded on a Jasco-720 spectropolarimeter equipped with a temperature-controlling unit. Cells of 0.1-cm pathlength were used throughout. Samples generally contained 10−40 μM of purified proteins in phosphate buffer saline (PBS), pH 7.4. Each independent CD measurement consisted of five consecutive scans. The final spectra were obtained by subtracting the solvent background from the measured spectra. To generate thermal denaturation profiles, the CD spectra of SAP or a SAP mutant were recorded at 5 °C intervals as the temperature was gradually raised from 15 to 80 °C.

RESULTS

SAP Mutants Are Deficient in Binding to Peptides Derived from SLAM. Six SAP mutations found in XLP patients (*23*−*26*) were generated by PCR and the corresponding mutant proteins, namely, L31P, Y54C, R55L, E67D, F87S, and

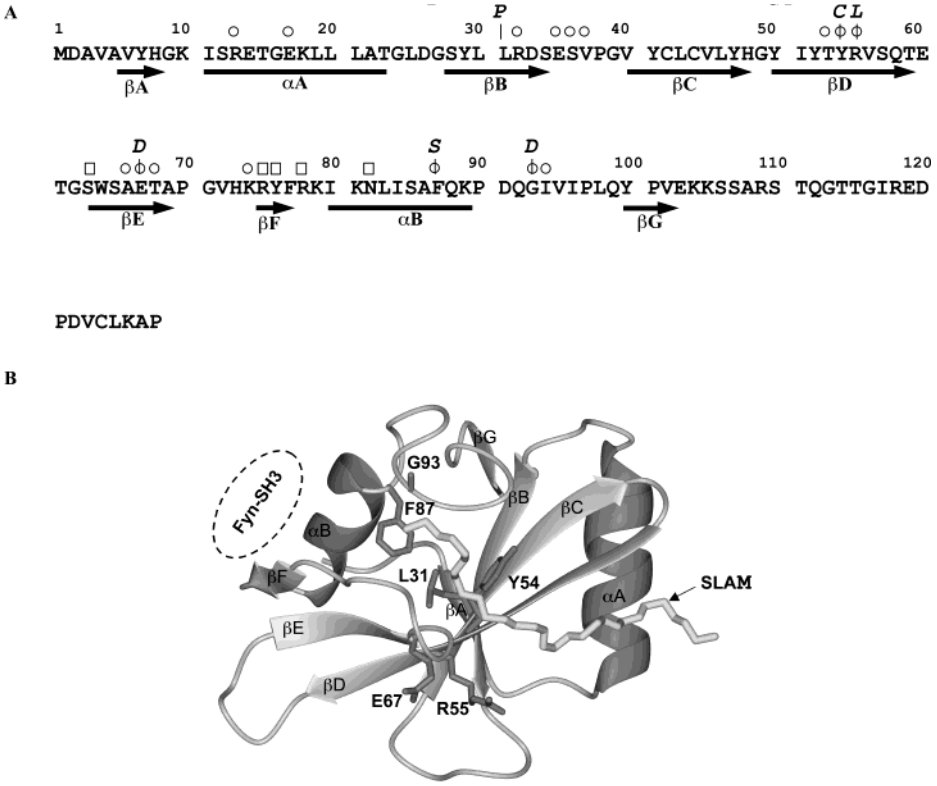

FIGURE 1: (A) Locations of missense mutations in the SAP/SH2D1A protein sequence. Secondary structural elements are labeled according to Huang et al. (*19*) below the protein sequence. Disease-causing amino acid substitutions (italicized) are listed above the naturally occurring residues. Residues mapped to the ligand-binding sites for SLAM and Fyn (*17*, *19*) are identified as open circles and squares, respectively. (B) Positions of missense mutations in a SAP−SLAM Y281 peptide complex. The structure shown was modified from Huang et al. (*19*). The SAP SH2 domain is represented as ribbons and the bound SLAM peptide is represented as backbone trace. Secondary structures and the mutated residues are labeled and the side chains of the mutated residues are identified as heavy atom traces. The Fyn−SH3 domain-binding surface (*17*) is schematically depicted by a broken circle.

G93D, were purified from *E. coli*. To our knowledge, these mutants have not been characterized biochemically or biophysically. It is interesting to note that all the mutations take place within the boundaries of the SAP SH2 domain, which encompasses residues 5−103 (Figure 1A), suggesting a pivotal role for the SH2 domain in SAP function. Since each mutation is sufficient to cause the XLP disease, it is likely that the physiochemical and/or binding properties of the mutant protein are altered.

The structures of several SAP SH2 domain−peptide complexes have been reported (*19*, *20*). Moreover, the Fyn SH3 domain-binding site in SAP was recently mapped out in the structure of a ternary complex of the Fyn SH3 domain, the SAP SH2 domain, and a SLAM peptide (*17*). The SH3-binding surface in SAP is composed mainly of residues from helix B (αB) and strand F (*β*F), and is opposite to that for the C-terminal residues of the SLAM peptide. The positions of the six mutations in the SAP SH2 domain−ligand complex are depicted in Figure 1B. To examine the affinities of purified SAP mutants for SLAM, two peptides, namely, SLAM-Y281 and SLAM-pY281, representing the sequence flanking the major SAP-binding site, Tyr281, in human SLAM (*6*, *18*), were synthesized and labeled with fluorescein. Fluorescence polarization studies demonstrated that SAP bound both the phosphorylated (-pY281) and the unphosphorylated (-Y281) SLAM peptides with high affinities as the corresponding binding curves reached saturation at approximately 10 μM SAP. In contrast, mutants R55L and F87S, especially the latter, exhibited significantly reduced binding to the same peptides (Figure 2). Dissociation constants (K_d) for the corresponding SAP and mutant peptide complexes, derived from fluorescence polarization, are listed in Table 1. While SAP interacts with the SLAM-pTyr281 or -Tyr281 peptide with K_d values in the submicromolar range, the majority of SAP mutants examined possess K_d values above 1 μM. The relative affinity of a mutant for peptide SLAM-pTyr281 was 30% or less of that for the wild-type (wt) SAP, whereas the relative affinity of a mutant for peptide SLAM-Tyr281 was approximately 40% or less of that for SAP. Therefore, missense mutations in the SAP SH2 domain lead to a marked decrease in binding to both the phosphorylated and unphosphoryalted SLAM peptides, which may result in impaired binding of the mutant proteins to the SLAM receptor both at its basal (unphosphorylated) and active (phosphorylated) states. As described below, the majority of the mutants are indeed deficient in binding to full-length SLAM and in signaling through the SLAM receptor in cells.

SAP Mutants Are Defective in Binding to Fyn SH3. Apart from interacting with SLAM, SAP is also capable of directly binding to Fyn. Because the latter event is mediated by the Fyn SH3 domain (*15*−*17*), we set out to examine whether SAP mutants are impaired in such an interaction. Purified Fyn SH3 was labeled with fluorescein and assayed for binding to SAP or a mutant by fluorescence polarization. As shown in Figure 3A, while wt SAP binds the Fyn SH3 domain with high affinity as evidenced by saturation of binding at approximately 25 μM SAP, the mutants, such as

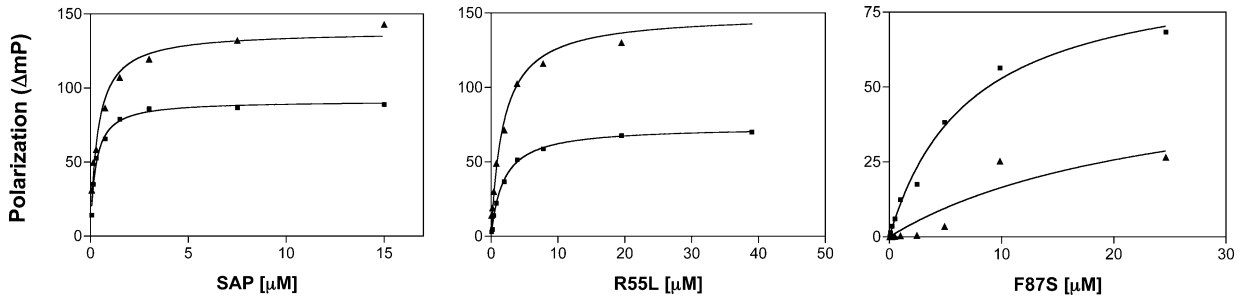

FIGURE 2: Binding of SAP mutants to SLAM peptides. Binding curves for SAP and mutants R55L and F87S to fluorescein-labeled SLAM-pY281 (squares) and Y281 (triangles) (see footnotes to Table 1 for sequence information). Relative polarization values (Y, Δmp) at various protein concentrations (X) from a representative experiment were fitted to the equation $Y = B_{max}X/(K_d + X)$, where B_{max} denotes maximum fluorescence polarization, to yield the corresponding K_d values using the software Prism 3.0. Average K_d values from at least two independent measurements are given in Table 1.

Table 1: Relative Affinities of SAP Mutants for Fluorescent Peptides[a] SLAM-pY281 and -Y281

	SLAM-pY281		SLAM-Y281	
protein	K_d (μM)[b]	rel affinity (%)	K_d (μM)[b]	rel affinity (%)
SAP	0.26 ± 0.03	100	0.60 ± 0.07	100
L31P	0.86 ± 0.08	30	1.40 ± 0.04	43
Y54C	7.18 ± 2.77	4	ND	ND
R55L	2.21 ± 0.29	12	1.96 ± 0.25	30
F87S	7.26 ± 1.16	4	20.17 ± 6.65	3
G93D	1.55 ± 0.17	17	11.94 ± 1.85	5

[a] The peptides contained the sequence fluorescein-GGR-*KSLTI(p)-YAQVQK*. The italicized sequence is derived from the Y281 locus in human SLAM. A GGR spacer was inserted between fluorescein and the peptide sequence to increase solubility and minimize end effects. [b] See legend to Figure 2 for the derivation of K_d values. ND: K_d not determined due to weak binding. Binding data not available for mutant E67D because of difficulties in purifying enough proteins to generate a saturation binding curve.

L31P and R55L, were significantly weaker in binding. Of the six mutants examined, all were found to exhibit reduced affinities for the Fyn SH3 domain. The corresponding K_d values ranged from 10 μM for mutant L31P to 27 μM for Y54C, compared to 2.7 μM for wt SAP (Table 2). The relative affinities of these mutants for the Fyn SH3 domain are estimated to be 25% or less of that for wt SAP (Table 2).

The substantial decrease in affinity for the Fyn SH3 domain observed on SAP mutants were further validated using full-length Fyn in a GST pull-down assay. SAP or a SAP mutant in GST fusion was examined for binding to Fyn expressed in human embryonic kidney (HEK) 293 cells. GST-SAP effectively brought down Fyn from 293 cell lysate, whereas the mutated proteins, in particular, Y54C, R55L, E67D and F87S, were significantly less efficient in precipitating Fyn (Figure 3B). This result agrees essentially with those obtained from fluorescence polarization studies using isolated Fyn SH3 domain (Table 2). Subtle differences, however, were observed between the affinities measured using the Fyn SH3 domain (Table 2) and those obtained using the intact Fyn kinase (Figure 3B). Because the Fyn SH2 domain may play an auxiliary role in the Fyn−SAP interaction (*15*), it is likely that the observed discrepancies originated from differences in binding to the Fyn SH2 domain for the mutants.

SAP Mutants Are Defective in Signaling through the SLAM Receptor. Functionally, the formation of a ternary complex of SLAM, SAP, and Fyn leads to SLAM phosphorylation and activation of downstream signaling events such as the recruitment of SHIP and the displacement of SHP-2 from the receptor (*6*, *14*−*16*). The ability of SAP mutants in SLAM signaling was examined in HEK 293 cells made to stably express SLAM. First, SLAM was immunoprecipated from HEK-SLAM cells that expressed SAP or a SAP mutant in the presence of Fyn. Cells that expressed SAP or Fyn alone and those that expressed neither SAP nor Fyn were used as controls. As shown in Figure 4A, both SAP and Fyn could be detected in anti-SLAM immunoprecipitates, indicating that SAP, SLAM, and Fyn are present in the same protein complex in cells (*15*−*17*). SLAM was phosphorylated on multiple tyrosines in cells expressing both SAP and Fyn, as demonstrated by the ladder pattern on an anti-pTyr blot (Figure 4A). SLAM phosphorylation was less strong in cells expressing Fyn alone, suggesting that the presence of SAP promotes the tyrosine phosphorylation of SLAM. The enhancement of SLAM phosphosphorylation, however, was not observed in cells expressing a SAP mutant. While the presence of L31P or G93D did not significantly affect SLAM phosphorylation by Fyn, expressing of any of the remaining four mutants depressed SLAM phosphorylation. This could be attributed to the absence or a decrease of SLAM-associated Fyn as well as to reduced Fyn kinase activity in cells containing these mutants (Figure 4A). Activation of Fyn, as reflected by its tyrosine phosphorylation, remained at the same level in cells expressing mutant Y54C, R55L, E67D, or F87S as in cells expressing Fyn alone (Figure 4B). In contrast, wt SAP and, to a lesser degree, mutants L31P and G93D, were able to activate Fyn by inducing its phosphorylation.

Most mutants, except for G93D, displayed significantly decreased binding to SLAM, which, in essence, agrees with results from the peptide binding studies (Table 1). Although SLAM expression appeared to be reduced in cells expressing either mutant Y54C, R55L, or E67D, it is unlikely that this would contribute significantly to the diminished binding observed for the three mutants because a roughly equal amount of SLAM was precipitated from each sample (Figure 4A, top panel). It should be pointed out that, although Tyr281 is the major SAP-binding site in SLAM, other sites such as Tyr307 and Tyr327 may also contribute to binding (*15*). This may explain the difference in binding affinity of a mutant measured in vitro using the SLAM-Y281 peptides (Table 1) from that obtained in vivo using the intact protein (Figure 4A).

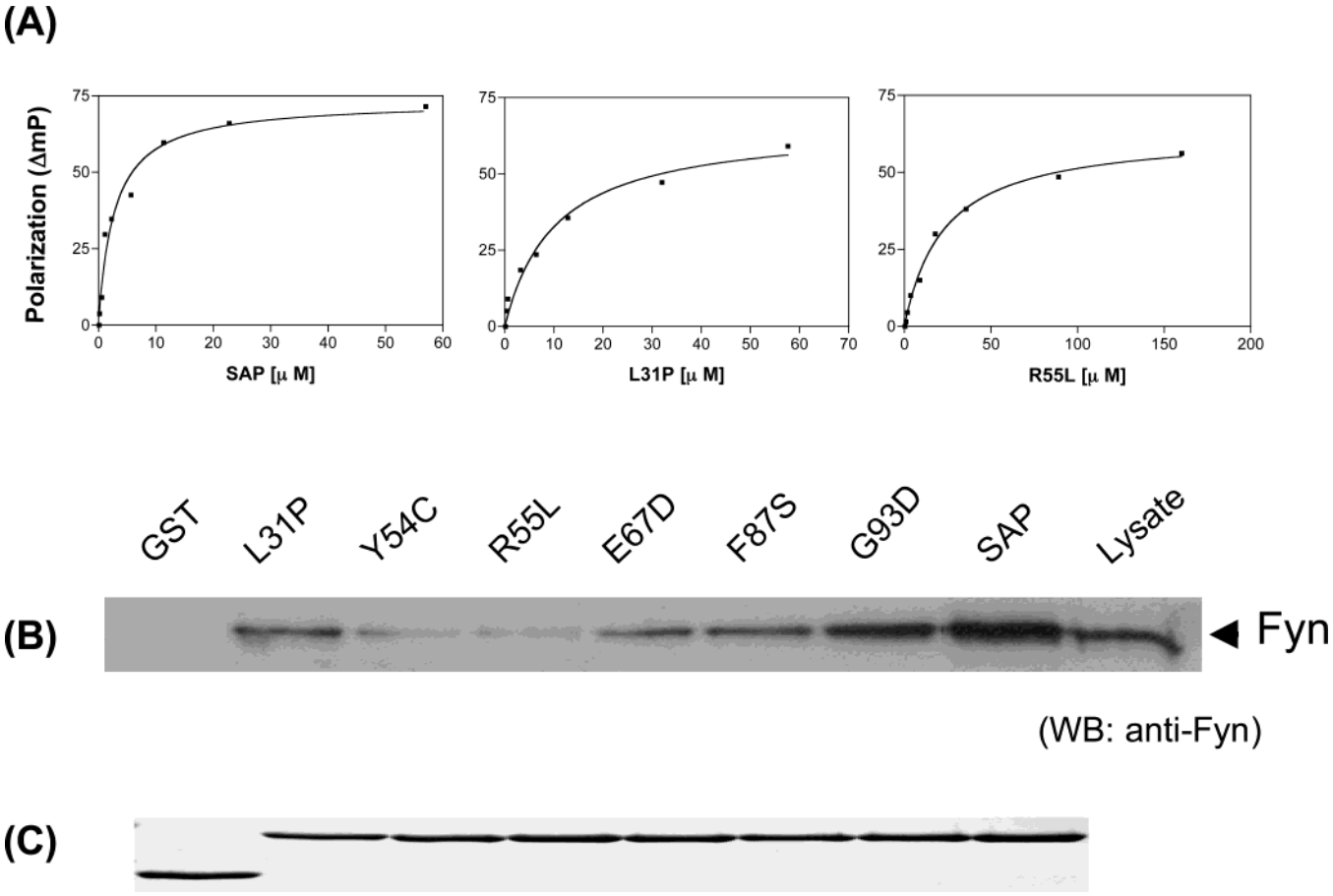

FIGURE 3: Deficient binding to Fyn and the Fyn SH3 domain for the SAP mutants. (A) Binding curves for SAP and mutants L31P and R55L to fluorescein-labeled Fyn SH3 domain generated by fluorescence polarization. Incremental amount of SAP or a mutant was titrated into a solution containing approximately 1.0 μM fluorescein-Fyn SH3 to yield the binding curves. (B) Binding of GST, GST-fusion SAP, or a mutant to Fyn expressed in HEK 293 cells. Approximately 15 μg GST fusion protein was used to pull down Fyn from 100 μL (~100 μg total protein) cell lysate for each lane. WB: Western blot; (C) Coomassie blue-staining of SDS−PAGE showing equal application of GST or a GST-fusion protein in each lane of (B).

Table 2: Relative Affinities of SAP Mutants for the Fyn SH3 Domain

protein	K_d (μM)[a]	rel affinity (%)
SAP	2.74 ± 0.54	100
L31P	10.27 ± 2.24	23
Y54C	27.54 ± 4.21	10
R55L	16.51 ± 3.52	17
E67D	12.89 ± 2.91	21
F87S	22.91 ± 2.44	12
G93D	12.42 ± 3.02	22

[a] The K_d values were determined based on fluorescence polarization data obtained using GST fused SAP or mutants and fluorescein-labeled Fyn SH3 domain. Reported values are averages over at least two independent measurements.

While SAP was capable of binding to unphosphorylated SLAM, binding of SHP-2 was dependent on SLAM-phosphorylation (Figure 4A). The presence of SAP blocked the interaction of SHP-2 with SLAM. However, only mutants L31P and G93D, which retained a significant binding affinity for SLAM, were capable of blocking the SHP-2−SLAM interaction. Mutants Y54C, R55L, E67D, and F87S, which exhibited markedly reduced affinities for SLAM, failed to do so (Figure 4A). The ability of a SAP mutant in preventing the recruitment of SHP-2 to the SLAM receptor is therefore coupled to its binding affinity for the receptor.

In a reciprocal experiment, anti-FLAG antibodies were used to immunoprecipitate SAP or a mutant from cells. The amount of SLAM coprecipitated with SAP or a mutant was determined by a Western blot using anti-SLAM antibodies. As shown in Figure 4C, wt SAP bound with SLAM regardless of Fyn, although the presence of Fyn appeared to enhance the SAP−SLAM interaction. Of the six mutants examined, L31P and G93D were more potent than the remaining four mutants in bringing down SLAM from cells, consistent with the results described in Figure 4A. Mutants Y54C, R55L, E67D, and F87S also displayed more pronounced reduction in binding to Fyn, compared to mutant L31P or G93D (Figure 4C). Interestingly, there appeared to be more Fyn associated with a mutant (Figure 4C) than with the SLAM receptor (Figure 4A). Because the same mutants (e.g., Y54C, R55L, E67D, and F87S) are ineffective in binding SLAM, it is possible that Fyn is sequestered by these mutants in the cytosol, resulting in hypophosphorylation of the SLAM receptor (Figure 4A).

Disease-Causing SAP Mutants Are Inherently Unstable. All mutations, except for mutant L31P, take place at the SLAM-binding groove of the SAP SH2 domain. It is conceivable that these mutations would directly affect interactions with SLAM, as shown to be indeed the case. However, the same mutants were also found to possess

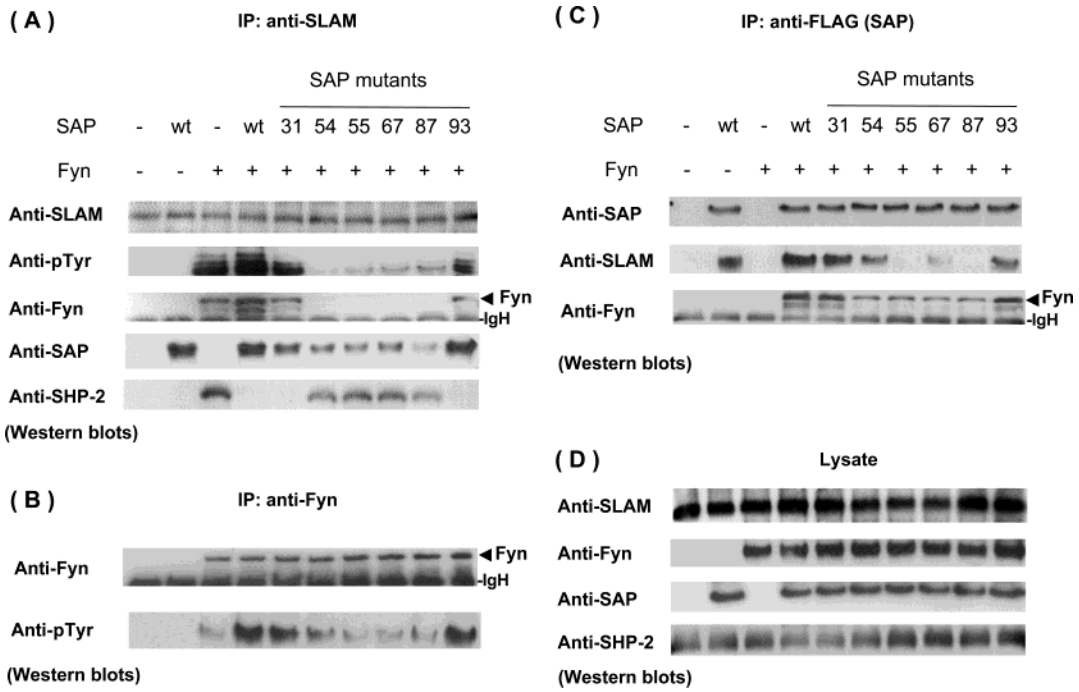

FIGURE 4: SAP mutants are impaired in signaling through the SLAM receptor. (A) HEK 293 cells stably expressing the SLAM receptor were either singularly transfected or cotransfected with pMES-Fyn and pFLAG-CMV2-SAP or a mutant. The corresponding cell lysate was subjected to anti-SLAM immunoprecipitation. Receptor-associated proteins were identified by sequentially blotting the membrane with antibodies against Fyn, SAP, and SHP-2. Phosphorylation levels of the receptor was probed with an anti-pTyr antibody. IgH, immunoglobulin heavy-chain. A minor band below that of Fyn is visible in some lanes of the anti-Fyn Western blots, which may represent degraded Fyn or a cross-reacting protein. (B) Fyn was immunoprecipitated from cells and Western blotted with anti-Fyn and anti-pTyr antibodies, respectively, to assay for Fyn expression and activation. (C) SAP was immunoprecipitated from the HEK-SLAM cells expressing SAP or a mutant using anti-FLAG antibodies. Proteins contained in the precipitates were examined in Western blots using antibodies against SAP, SLAM and Fyn, respectively. (D) Western blots of cell lysate to show the levels of expression for SLAM, Fyn, SAP/mutant, and SHP-2 in each sample. SLAM present on the cell surface was biotinylated, precipitated using streptavidin beads, and blotted using anti-SLAM antibodies.

significantly reduced affinities for Fyn or the Fyn SH3 domain. Since none of the mutated residues are directly involved in binding Fyn (Figure 1B), other factors may contribute to this phenomenon. Our observation that the mutant proteins are generally more prone to proteolysis than wt SAP during the purification process (data not shown) suggests that they may be improperly folded. To explore this possibility, SAP or a mutant was cleaved off the GST moiety and subjected to thermal denaturation as monitored by CD spectroscopy. As shown in Figure 5A, SAP assumed a CD spectrum indicative of a mixture of α-helices and β-sheets at 20 °C, consistent with its determined structure (*19*, *20*). There was negligible loss of secondary structures as the temperature was raised to 50 °C (Figure 5A), suggesting that SAP is stable and resilient to thermal denaturation. Further elevation in temperature to 60 °C, however, induced a rapid disintegration of the protein's structure (Figure 5A). The corresponding denaturation curve shows a sharp structural transition between 55 and 60 °C, which coincides with a melting temperature of 58 °C (Figure 5B). The cooperative nature of denaturation indicates that SAP is folded properly at room temperature.

Notwithstanding these observations, the SAP mutants exhibited strikingly different thermal denaturation patterns (Figure 5C−F). A gradual loss of ordered structures was observed for mutant Y54C between 20 and 60 °C. The corresponding denaturation curve (Figure 5D) showed no discernible transition point, indicative of lack of cooperativity in unfolding. Some mutants, such as E67D, exhibited cooperative thermal denaturation profiles similar to that for wt SAP. However, these mutants possess greatly reduced melting temperatures (Figure 5E,F). An abrupt structural transition was observed for mutant E67D at 40−45 °C, compared to around 60 °C for SAP. Therefore, a relatively conservative mutation from a Glu to an Asp residue in SAP leads to a drastic reduction in thermal stability for the protein.

All six mutants examined displayed significant defects in folding. As seen in Table 3, the differences in melting temperatures between SAP and a mutant range from 6.3 °C for R55L to approximately 14 °C for E67D. On the basis of their denaturation profiles, these folding-defective mutants can be categorized into two subgroups. The first group, including mutants L31P, Y54C, and F87S, unfold in an uncooperative manner, whereas the second group, consisting of mutants R55L, E67D, and G93D, unfold cooperatively but at markedly reduced melting temperatures (Table 3).

DISCUSSION

Dysfunction of signaling proteins as a result of mutations often underlies human diseases. While most mutations occur in the catalytic domains of kinases and phosphatases involved in cellular signaling events (*27*), some are found in protein-interaction domains such as SH2. Similar to the detrimental effects arisen from mutations in the SAP SH2 domain, which are responsible for the onset of the XLP syndrome, mutations in the SH2 domain of the Bruton's tyrosine kinase (BTK) are associated with the X-linked agammaglobulinemia, another disease of the immune system (*28*). Although it is

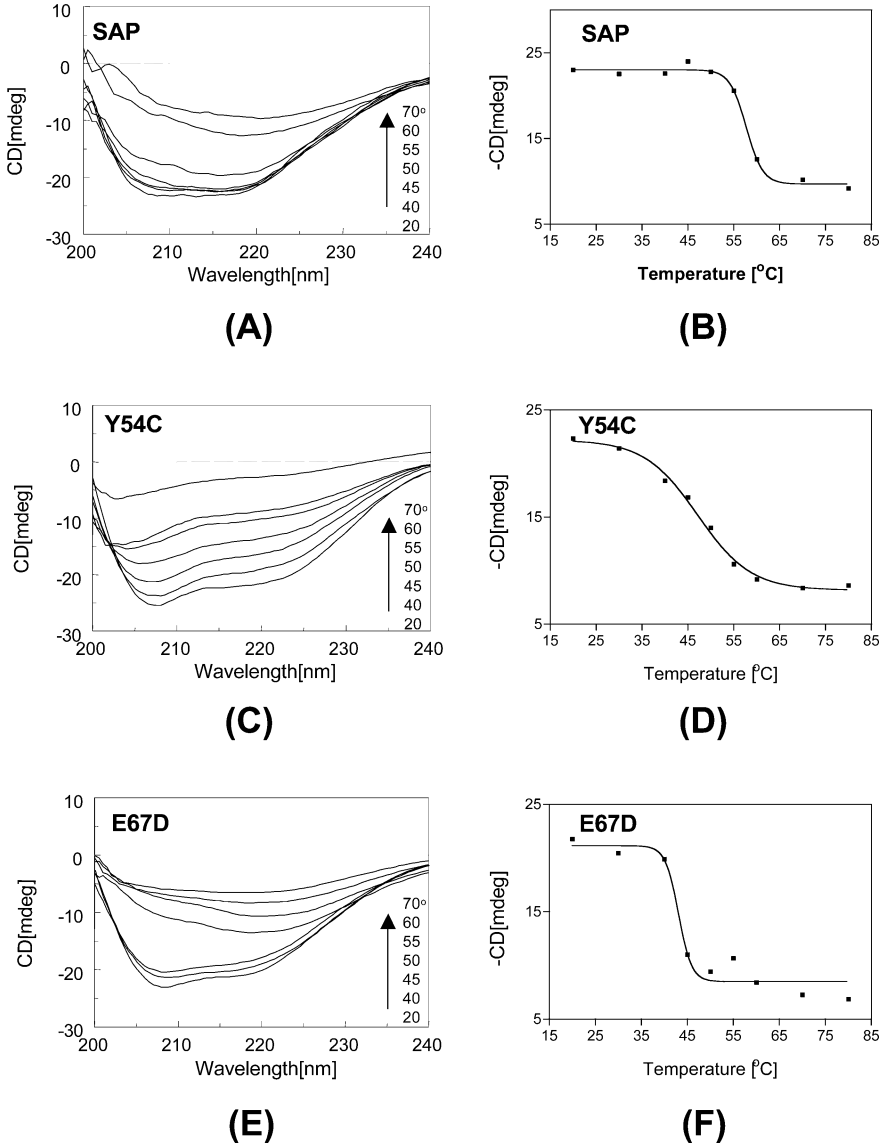

FIGURE 5: Thermal denaturation of SAP and mutants. CD spectra of SAP (A), Y54C (C), and E67D (E) at various temperatures as indicated on the diagram and their corresponding unfolding curves (B, D, and F) generated by plotting the CD values (converted to positive numbers) at 218 nm against temperature (°C).

Table 3: Locations of Missense Mutations in SAP and Melting Temperatures Determined for the Corresponding Mutants

residue	location	specificity[a]	mutant	T_m (°C)	ΔT_m (°C)[b]	group
Leu31	βB	NB	L31P	45.6 ± 0.8	−12.2	I
Tyr54	βD	C-binding (SLAM)	Y54C	48.2 ± 0.7	−9.6	I
Phe87	αB	C-binding (SLAM)	F87S	50.5 ± 0.6	−7.3	I
Arg55	βD	pY-binding (SLAM)	R55L	51.5 ± 0.3	−6.3	II
Glu67	βE	C-binding (SLAM)	E67D	44.2 ± 0.9	−13.6	II
Gly93	BG-loop	C-binding (SLAM)	G93D	49.1 ± 0.6	−8.7	II

[a] Residues involved in binding the SLAM peptide were identified according to Huang et al. (*19*). See also Figure 1B. NB, not directly involved in binding to either SLAM or Fyn. [b] T_m, melting temperature derived from a corresponding thermal denaturation curve. T_m for wild-type SAP was 57.8 °C. $\Delta T_m = T_m$ (mutant) − T_m (SAP).

not completely understood how SAP regulates the signaling and activation of lymphocytes, it has been shown to interact with SLAM, a co-stimulatory molecule in the activation of the T cell antigen receptor, and with Fyn, a protein tyrosine kinase important for immunoreceptor signaling (*6*, *14*−*17*). Interestingly, these two interactions are mutually dependent: binding of SAP promotes the recruitment of Fyn to SLAM, leading to the tyrosine phosphorylation of SLAM, which, in turn, reinforces the SAP−SLAM interaction (*15*). In this regard, SAP functions as an adaptor by bridging a SLAM−Fyn interaction. The determination of SAP structure in complex with peptides derived from the SLAM receptor (*19*, *20*) and, more recently, with the Fyn SH3 domain (*17*), has provided a molecular basis for SAP function. Although small in size, SAP can simultaneously engage SLAM and Fyn using distinct binding surfaces (*17*). Binding of SAP to Fyn not only serves to couple the latter to SLAM, it is also thought to activate the Fyn kinase activity by releasing an inhibitory intramolecular interaction involving the Fyn SH3 domain (*17*). Although SAP associates with SLAM constitutively in cells, tyrosine phosphorylation of a SLAM peptide increases its affinity for SAP by 5-fold (*18*). Apparently, the ability of SAP to interact with both SLAM and Fyn with

high affinity and specificity is pivotal to the normal function of SAP, and compromising this ability may cause disease.

Missense SAP mutations associated with the XLP syndrome can provide a wealth of information on the pathogenesis of the disease. Our studies on a panel of six SAP mutants uncovered a number of defects. First, most mutants display significantly reduced affinities for SLAM and/or SLAM-derived peptides. Second, they are much less efficient in binding to Fyn or to the SH3 domain of Fyn. Third, signaling activities of these mutants through the SLAM receptor are largely impaired. And last, all mutants are defective in folding as manifested by their significantly compromised thermal stability.

It is likely that defects in protein folding are related to and are at least partly responsible for the observed deficiencies in ligand-binding for the mutants. Although the detailed molecular mechanism of misfolding of a SAP mutant awaits further investigation, our studies provide compelling evidence that directly correlates misfolding with loss of function for a disease-associated protein. While the mutants can be arbitrarily divided into two groups based on their distinct thermal denaturation profiles, they share some common characteristics. First, most mutations occur at sites of regular secondary structures, especially on β-strands (Figure 1). These mutations are expected to disrupt the packing of the central β-sheet of the SAP SH2 domain that is essential for the integrity of the protein (*19*, *20*). Second, except for mutant E67D, all mutations result in drastic alterations of the side chain chemistry of the residues involved. These alterations can affect local structures and impact directly or indirectly on ligand binding.

What are the consequences of an improperly folded protein? It is reasonable to assume that an improperly folded mutant be inherently less stable than the wild type, and would undergo proteolysis more rapidly inside the cell. This is indeed the case for several SAP mutants which have been shown to have much reduced half-lives in cells compared with wt SAP (*8*). A low level of protein may contribute to dysfunction of SAP in SLAM signaling. A mutant that is misfolded can also compromise its ability to bind to the SLAM family of receptors and/or to Fyn, assuming that only the native fold of the protein is competent in binding. Taken together, our results suggest that the normal function of SAP requires its selective and high-affinity binding to Fyn and SLAM, and that mutations, which either directly interfere with binding or indirectly affect binding due to improper protein folding, underlie the XLP syndrome.

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