Missense mutations in \textit{SH2D1A} identified in patients with X-linked lymphoproliferative disease differentially affect the expression and function of SAP

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Abstract

\textit{X-linked lymphoproliferative disease (XLP) is an immunodeficiency resulting from mutations in \textit{SH2D1A}, which encodes signalling lymphocytic activation molecule (SLAM)-associated protein (SAP). In addition to SLAM, SAP associates with several other cell-surface receptors including 2B4 (CD244), Ly9 (CD229), CD84 and NTB-A. SAP contains a single src-homology-2 domain and acts as an intracellular adaptor protein by recruiting the protein tyrosine kinase FynT to the cytoplasmic domains of some of these receptors, which results in the initiation of specific downstream signal transduction pathways. XLP is likely to result from perturbed signalling through one or more of these SAP-associating receptors. In this study, we identified missense (Y54C, I84T and F87S) and insertion (fs82 \rightarrow X103) mutations in four different kindreds affected by XLP. Each mutation dramatically reduced the half-life of SAP, thus diminishing its expression in primary lymphocytes as well as in transfected cell lines. Interestingly, although the Y54C and F87S mutations compromised the ability of SAP to associate with different receptors, the I84T mutation had no effect on the ability of SAP to bind SLAM, CD84 or 2B4. However, signalling downstream of SLAM was reduced in the presence of SAP bearing the I84T mutation. These findings indicate that, irrespective of the type of mutation, signalling through SAP-associating receptors in XLP can be impaired by reducing the expression of SAP, the ability of SAP to bind surface receptors and/or its ability to activate signal transduction downstream of the SLAM–SAP complex.}

Introduction

\textit{X-linked lymphoproliferative disease (XLP) is an inherited immunodeficiency characterized by extreme sensitivity to infection with the human herpes group virus EBV (1–3). The gene mutated in XLP is \textit{SH2D1A} which encodes the signalling lymphocytic activation molecule (SLAM)-associated protein (SAP), a small src-homology-2 (SH2) domain-containing protein that interacts with members of the CD2 superfamily of cell-surface receptors (4–6). For example, SAP binds to SLAM (CD150), 2B4 (CD244), CD84, Ly9 (CD229) and NTB-A, and its interaction with the cytoplasmic domain of these receptors is mediated via a unique tyrosine-based motif (TxxYxxV/I) (3, 4, 7–14). The interaction between SAP and SLAM or 2B4 facilitates the recruitment of the src family kinase FynT, which subsequently activates downstream signal transduction pathways (15–17). Consequently, altered signalling via SAP-associating cell-surface molecules may contribute to some of the immunological defects observed in XLP. Indeed, activation of the cytotoxic function of NK cells from XLP patients through ligation of 2B4 or NTB-A is selectively impaired, while the ability of these cells to lyse target cells when activated through receptors that do not associate with SAP is intact (9, 18–20).}

\textit{Mutations in \textit{SH2D1A} have been identified in >200 XLP patients (3–6, 21–23). The majority of these are gross...}
alterations such as the deletion of the entire \textit{SH2D1A} gene or individual exons or mutations in exon/intron splice sites. The remaining alterations are point mutations that result in either the introduction of a premature stop codon or missense mutations resulting in amino acid substitutions, predominantly within conserved residues of the \textit{SH2} domain of SAP (3–6, 21–23). To date, the effects of only a few of the previously identified missense mutations on the expression and function of SAP have been investigated. In some of these studies, it has been found that mutations in SAP can affect protein stability or the ability of SAP to associate with SLAM family receptors or with FynT (12, 24–28).

In this study, we have examined four different kindreds with XLP and identified the disease-causing \textit{SH2D1A} mutations in each case. We identified three missense mutations (Y54C, I84T and F87S) and one insertion/frameshift mutation (fs82 → X103), two of which have not been previously identified in XLP patients (I84T and fs82 → X103). Each mutation dramatically reduced the half-life of SAP, thereby diminishing the level of expression in primary cells, as well as in stably or transiently transfected cell lines. Interestingly, whereas the Y54C and F87S mutations compromised the ability of SAP to associate with SLAM, 2B4 and CD84, the I84T mutation had no effect on this event. However, the I84T mutation was impaired in its ability to facilitate signalling downstream of SLAM. In line with these observations, NK cells from patients with the different missense mutations could not be induced to lyse target cells in response to ligation of 2B4. These findings are consistent with previous studies and suggest that, irrespective of the type of mutation, signalling through SAP-associating receptors in XLP will be impaired through one or more simultaneously acting mechanisms—(i) reduced expression of SAP, (ii) reduced ability of SAP to bind to SLAM family receptors and (iii) reduced ability to activate signal transduction downstream of the SLAM receptor–SAP complex. This would also explain why there is no correlation between the type of \textit{SH2D1A} missense mutation and the disease phenotype (21).

\section*{Methods}
\subsection*{Antibodies}
The following antibodies were used in this study: PE-anti-CD3 and anti-CD56 mAbs (Becton Dickinson, San Jose, CA, USA); purified anti-CD16 mAb, PE-anti-CD84 mAb and streptavidin (SA)-conjugated to PerCP (BD PharMingen, San Diego, CA, USA); anti-Fyn mAb (BD Transduction Laboratories); purified anti-CD84 mAb (clone 152-1D5, NeoMarkers, Fremont, CA, USA); purified and PE-conjugated anti-2B4 mAb (c1.7, Beckman-Coulter) (29); biotinylated anti-SLAM mAb (clone A12, eBioscience, San Diego, CA, USA) (30); purified anti-FLAG mAb (M2, Sigma–Aldrich); anti-phosphotyrosine mAb (4G10, Upstate Biotechnology Inc., Lake Placid, NY, USA); anti-myc mAb (9E10), anti-IC3 mAb (clone 3A5) and rabbit polyclonal anti-SHP-2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); HRP-conjugated donkey anti-rabbit IgG and anti-mouse IgG antiserum (Amersham-Pharmacia, Castle Hill, Australia). Rabbit anti-human SAP polyclonal antiserum was generated by immunizing rabbits with a synthetic peptide corresponding to the carboxy terminus of human SAP (18).

\subsection*{Monoclonal cells and lymphocyte purification}
Mononuclear cells (MNCs) were isolated from peripheral blood of normal healthy donors and XLP patients by centrifuging diluted whole blood over Ficoll–Paque (Amer- sham Biosciences, Uppsaal, Sweden). The clinical manifestations of some of the XLP patients examined in this study, as well as several aspects of lymphocyte development, have been described previously (31–33). The effects of \textit{SH2D1A} mutations on expression of SAP in patients XLP#1, 2 and 3 have been published (14, 34). However, the biochemistry underlying these effects has not been previously examined. NK cells were purified from MNCs using CD56 MACs beads and an AutoMACS cell separator. NK cells were also isolated by cell sorting following labelling of MNCs with anti-CD3 and anti-CD84 mAb, and collecting CD3+CD56+ cells. T cell blasts were generated by stimulating MNCs (10^5 m^{-1}) with PHA (5 μg ml^{-1}; Sigma Chemical Co., St Louis, MO, USA) and IL-2 (20 U ml^{-1}; Endogen, Woburn, MA, USA). Cell lines used in this study were the human T cell line F2F7, the murine pro-B cell line BaF/3, mouse mastocytoma cell line P815 and human 293T cells. All cell lines were cultured at 37°C in 5% CO_2 in RPMI-1640 tissue culture medium (JRH Biosciences) supplemented with 10% foetal bovine serum, penicillin and streptomycin and L-glutamine.

\subsection*{Expression of SAP}
To determine the expression of SAP, whole-cell lysates were prepared by solubilizing cells in ice-cold lysis buffer [10 mM Tris–HCl (pH 7.8), 0.1% NP-40, 150 mM NaCl and protease inhibitors] (10). Cell lysates were electrophoresed through 15% acrylamide gels containing 0.1% SDS and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Bedford, MA, USA). Membranes were probed with antibodies against SAP, SHP-2 or lck followed by HRP-conjugated donkey anti-rabbit IgG or anti-mouse IgG antiserum. The membranes were developed using enhanced chemiluminescence (Pierce, Rockford, IL, USA) and autoradiography.

\subsection*{Cloning and construction of expression plasmids}
BaF/3 cell lines expressing N-terminal FLAG-tagged full-length human 2B4 or CD84 alone or co-expressing CD84 and wild-type (WT) SAP have been previously described (7, 10, 14). The open reading frame of human SLAM, minus the leader sequence, was amplified from the pSURS/1 plasmid (30; provided by Rene de Waal Malefyt, Schering Plough Biopharma, Palo Alto, CA, USA) using as primers 5′-GCAATGCATCGATC GC ATG ATG AAC TGC CCA AAG-3′ (RI restriction site underlined) and 3′-GCA TGC GAC TGC ATG ATG AAC TGC CCA AAG (Cltal site underlined) and 3′-GCA TGC GCGGCC TCA TCT TCT GGA AGT GTC AC (NolI site underlined). The amplified product was digested with Clal and NolI and cloned into pMSEX-neo downstream of the FLAG epitope (DYKDDDDK) and the CD8 leader sequence (7, 10), yielding a FLAG–SLAM construct. Sequences containing mutated \textit{SH2D1A} were amplified from cDNA prepared from MNCs isolated from different XLP patients using as primers 5′-GAA GAA GGATCC GCC ATG GAC GCA GTG GCT G (BamHI restriction site underlined) and 3′-GCA ATC TCGA GATC TGG CTC AGT GAC GCA ATC (EcoRI restriction site underlined); 7). These primers are located at the open reading frame of WT
SAP. To amplify a sequence encoding a mutant SAP due to a base pair insertion, resulting in a frameshift at amino acid 82, the above 5′ primer was used in combination with 3′-GAA TTC ACT GGA TAC TGC AGA GG (EcoRI restriction site underlined). This reverse primer corresponded to nucleotides 290–306 and terminates immediately upstream of the premature stop codon induced by this frameshift mutation (amino acid 103). The PCR-amplified products were digested with BamHI and EcoRI, and then cloned into the pMX-puro retroviral expression vector upstream of an in-frame sequence encoding the c-myc epitope, generating a mutant SAP myc construct (7). This vector also contained a cDNA encoding enhanced green fluorescent protein (eGFP) preceded by an internal ribosome entry site (IRES) element (10). The nucleotide sequences of all constructs were confirmed by automated DNA sequencing (performed by SUPAMAC, University of Sydney, Australia). A plasmid containing human Fyn was generously provided by Sarah Courtneidge (Van Andel Research Institute, Grand Rapids, MI, USA).

Cell transfection
To generate stable transfectants, pMX expression constructs were packaged using the Phoenix cell line (35) and virus used to infect the mouse pro-B cell line BaF/3 (7). Infected cells were collected using either puromycin (SAP) or neomycin (CD84, 2B4, SLAM), followed by cell sorting. Expression of CD84, 2B4 or SLAM by the transfected cells was assessed by flow cytometry using specific mAbs. Transduction of cells with SAP-encoding plasmids was inferred from eGFP fluorescence (10, 14). WT and mutant versions of SAP were also transiently expressed in 293T cells, either alone or in combination with SLAM, Fyn or both, using Lipofectamine (GIBCO Life Technologies).

Immunofluorescent staining
Cell lines were incubated with PE-conjugated control IgG, or anti-CD84 mAb, anti-2B4 mAb or biotinylated anti-SLAM mAb followed by SA–PerCP for 20–30 min on ice, before washing with cold PBS containing 0.1% BSA and 0.1% sodium azide. Data were acquired on a FACSscan flow cytometer and analysed using CellQuest software (Becton Dickinson).

Immunoprecipitation, SDS-PAGE and western blotting
Stably transfected BaF/3 cells or PHA blasts were re-suspended in PBS and were either untreated or treated with 100 μM sodium pervanadate prepared in 0.1% H2O2 (Sigma Chemical Co.) for 5 min at room temperature (7), and then solubilized in ice-cold lysis buffer (see Expression of SAP). The 293T cells were harvested 24–48 h after transient transfection, and lysed as above. SAP or SLAM was immunoprecipitated from lysates using anti-SAP polyclonal antiserum, anti-myc (SAP) or anti-FLAG mAb (SLAM) adsorbed onto Protein A–Sepharose beads (Pharmacia Biotech, CA, USA). Precipitated proteins were electrophoresed through acrylamide gels containing 0.1% SDS and transferred to PVDF membranes (Millipore Corporation). Membranes were probed with antibodies against phosphotyrosine, 2B4, CD84, FLAG, SAP or myc, followed by HRP-conjugated donkey anti-rabbit IgG or anti-mouse–IgG antiserum. The membranes were developed as described above (see Expression of SAP).

Determining the half-life of mutant SAPs
Baf/3 cells transfected with WT or mutant SAPs were harvested, washed and re-suspended in PBS at 106 ml−1. NHS-biotin (Pierce), prepared in dimethyl sulphoxide (DMSO), was added to a final concentration of 200 μg ml−1. When prepared in DMSO, this biotin ester is membrane permeable, and thus biotinylates intracellular proteins. The cells were incubated at room temperature for 1 h, with occasional mixing. After this time, the cells were washed three times with sterile PBS before being cultured in media. Aliquots of labelled cells were removed after different time periods and solubilized in 1% NP-40 lysis buffer. SAP was immunoprecipitated with anti-SAP antibody, electrophoresed through 15% polyacrylamide gels and transferred to PVDF. Total SAP was detected with anti-SAP antibody, while biotinylated SAP was detected with SA-conjugated HRP.

NK cell cytotoxicity assay
Cytotoxicity of NK cells was determined using a europium-release assay (36). Purified NK cells were cultured for 24 h in the presence of IL-2 (50 U ml−1). P815 target cells were harvested, re-suspended at 2 × 106 ml−1 and labelled with BATDA (Wallac, Turku, Finland) for 30 min at 37°C. After this time, P815 target cells were washed with RPMI-1640 media supplemented with 125 μM sulphhydrylrezone (Sigma Chemical Co.) to prevent leakage of the dye. NK cells and target cells were added to the wells of a 96-well round-bottom plate at an effector:target cell ratio of 2.5:1, in the absence or presence of a control mAb (anti-CD20), anti-2B4 mAb or anti-CD16 (5 μg ml−1), briefly centrifuged, and then incubated at 37°C for 2 h. Labelled P815 cells were also incubated with media or 10% NP-40 prepared in distilled water (BDH Laboratory Supplies, UK) to determine spontaneous lysis and maximum lysis, respectively. Following the incubation, 20 μl of the supernatant was transferred to a 96-well flat-bottom plate containing 200 μl of europium solution (Perkin-Elmer). The plate was shaken for 15 min before the fluorescence was measured using a Wallac Victor 1420 multilabel counter (Perkin-Elmer). The percentage of lysis was determined using the following formula: (experimental value – spontaneous lysis)/(maximum lysis – spontaneous lysis) × 100.

Results
Identification of SH2D1A mutations in different XLP patients
Disease-causing mutations in SH2D1A are predicted to affect signalling through SAP-associating receptors by disrupting the phosphotyrosine-binding site, decreasing protein stability or altering the effector function of the C-terminal domain (12, 37). Indeed, several mutations have been found to reduce the amount of SAP detected in XLP MNCs (38) or in cell lines transiently transfected with plasmids encoding mutant SH2D1A cDNA (24, 25). Based on this, several groups have reported that screening for the expression of SAP in activated MNCs can diagnose XLP (38–40). By using this approach, we identified 10 different XLP patients from four unrelated families (Family A: patient XLP#1; Family B: patients XLP#2 and 3;
Family C: patients XLP#4, 5, 6, 7, 8 and 16, and Family D: patient XLP#18. As shown in Fig. 1(a and b), the amounts of SAP in activated MNCs from patients from each of these four different families were reduced and essentially undetectable compared with the normal donors.

The disease-causing mutations present in these patients were determined next. Analysis of SH2D1A cDNA isolated from activated MNCs revealed the presence of missense mutations in three of the families and an insertion in the fourth family (schematically presented in Fig. 1c). One patient (Family A: XLP#1) had a single A → G mutation at nucleotide 460 in exon 2, resulting in a change at amino acid position 54 from tyrosine to cysteine (Y54C). Two patients (Family B: XLP#2 and 3) had a single T → C mutation at nucleotide 559 in exon 3, resulting in a change at amino acid position 87 from phenylalanine to serine (F87S). These two mutations (i.e. Y54C and F87S) have been previously described (14, 21). However, novel mutations were identified in two additional kindreds—patients XLP#4, 5, 6, 7, 8 and 16 from Family C had a single T → C mutation at nucleotide 551 in exon 3, resulting in a change at amino acid position 84 from isoleucine to threonine (I84T). These three missense mutations were all positioned within residues that are conserved in the SH2 domain of SAP (2). In another patient (Family D: XLP#18), an insertion of an adenosine residue at nucleotide position 544 in exon 3 resulted in a frameshift occurring at amino acid number 82, yielding a different amino acid sequence comprising 29 residues, followed by a premature stop codon being introduced at codon/arnino acid 103 (fs82 → X103). Thus, the protein encoded by this transcript differed from WT SAP from amino acid 83 to 102.

Over-expression of mutant SAP fails to improve expression

The reduced expression of SAP observed for the XLP patients relative to the WT controls may reflect impaired or altered activation of XLP MNCs (41). To evaluate this possibility, expression plasmids encoding WT or mutant SAP, as well as an IRES-eGFP element (10, 14), were generated and used to transduce the mouse pro-B cell line BaF/3. Following selection and cell sorting, >80–90% of cells had been transduced and expressed the introduced cDNA, as revealed by flow cytometric detection of eGFP (Fig. 2a). When lysates were prepared from these transfectants, it was found that the Y54C, F87S and I84T mutations we identified in the different XLP patients greatly reduced the amount of SAP expressed (Fig. 2b, upper panel), thereby confirming that these missense mutations significantly affect protein expression.

The anti-SAP polyclonal antibody used in our studies recognizes the 26 C-terminal amino acids of SAP (10, 14, 18). Due to the frameshift induced by the fs82 → X103 mutation, the resultant protein would not be detected by this antibody. Thus, to assess whether a protein is encoded by this mutant cDNA, it was engineered to include a C-terminal myc epitope tag, upstream of a stop codon, similar to our previous studies of WT SAP (10, 14, 18). When lysates from BaF/3 cells transfected either with a retroviral plasmid containing WT SAP.myc or with the SAP.fs82 → X103.myc construct were assessed for SAP using an anti-myc mAb, a protein of the appropriate size was only detected in cells expressing WT SAP.myc (Fig. 2c, upper panel). The anti-myc mAb was found to be less sensitive than the anti-SAP polyclonal antibody for detecting SAP by western blotting (see Fig. 2d). In order to improve detection of the mutant SAP, lysates of transfected cells were immunoprecipitated with anti-myc antibodies, and the presence of the precipitated proteins was assessed by western blotting using anti-myc mAbs. In this experiment, anti-myc mAb immunoprecipitated a protein only from those cells that had been transduced with WT SAP.myc plasmid (Fig. 2c, lower panel). Thus, similar to the single missense mutations identified in the other patients, this frameshift mutation also severely affected protein expression, in this case essentially preventing the expression of SAP.

To further extend our studies into the effect of these mutations on protein expression, 293T cells were transiently transfected with WT and mutant SAP plasmids, and the expression of SAP was determined by western blotting ~48 h later, a time determined to result in maximal SAP expression in this system (data not shown). This was performed so as to be able to examine the expression of SAP earlier following transfection, and to eliminate any effect that the antibiotic selection process may have on protein expression in stably transduced BaF/3 cells. Monitoring eGFP expression revealed comparable transduction efficiencies of all expression plasmids encoding WT or mutant SAP (data not shown). Assessment of the expression of SAP.myc with anti-myc mAb revealed abundant expression of WT SAP in lysates of transfected 293T cells (Fig. 2d, lane 1, upper panel). In contrast, the mutant forms of SAP were undetectable (Fig. 2d, lanes 2–5, upper panel). When the membranes were probed with the anti-SAP polyclonal antibody, abundant levels of WT SAP were detected (Fig. 2d, lane 1, middle panel), but only low levels of mutant Y54C and I84T SAP were observed, and the F87S mutant remained below the limit of sensitivity of this assay (Fig. 2d, lanes 2–4, middle panel). Not surprisingly, the fs82 → X103 mutant was not detected with the anti-SAP

![Fig. 1.](image)

Identification of novel mutations in SH2D1A that affect the expression of SAP. (a and b) MNCs were isolated from healthy donors (HD) or from each of the different patients and then cultured with PHA and IL-2. After 5–8 days, non-adherent cells were collected and solubilized in 1% NP-40 lysis buffer. Expression of SAP in lysates from patients in (a) Families A, B and C or (b) Family D was determined by SDS-PAGE and western blotting of whole-cell lysates using anti-SAP polyclonal antiserum (upper panel). Membranes were also probed with (a) anti-SHP-2 or (b) anti-lck antibody to demonstrate equal loading of cellular proteins (lower panel). (c) Schematic representation of the position of the different missense mutations within SAP in the affected patients. The filled grey region corresponds to the SH2 domain.
antibody due to the absence of the reactive epitope in this protein. Thus, these missense mutations severely reduce the expression of SAP not only in primary cells but also in transfected cells.

Mutations in SH2D1A dramatically reduce the half-life of SAP

The majority of SH2D1A mutations that have been previously characterized biochemically has been found to reduce the half-life of SAP (24, 42, 43). To determine the effect of the Y54C, F87S and I84T mutations on the half-life of SAP, BaF/3 cells stably expressing WT or mutant SAP were biotinylated such that intracellular proteins could be detected. The decay in the abundance of biotinylated SAP was then examined over a 24-h period. Labelled WT SAP could be detected at all time points examined, indicating it had a half-life of ~20 h (Fig. 3a, left panels), a value consistent with previous studies (24, 42). On the other hand, although the mutant SAPs could be detected immediately following biotinylation (0 h, anti-SAP i.p.), the ability to detect all three mutant proteins was dramatically reduced within 5 h of culture (Fig. 3b–d, left panels). While the amount of mutant SAP expressed by the transfectants was substantially less than that of WT SAP (Figs 2b and 3b–d, right panel, WCL lane), reasonable amounts of mutant SAP could be immunoprecipitated from the transfectants (Fig. 3b–d, right panels). Based on this, it is likely that the inability to detect biotinylated mutant SAPs resulted from accelerated turnover, and therefore a shortened half-life, rather than reduced detection following immunoprecipitation. Thus, similar to some other missense mutations in SH2D1A, those identified in our study also affect SAP expression by reducing its half-life.

Mutations in SH2D1A differentially affect the ability of SAP to associate with the SLAM family of cell-surface receptors

To date, SAP has been reported to associate with the cell-surface receptors SLAM, 2B4, CD84, Ly9 and NTB-A—the ‘SLAM family’ (4, 7–10, 14). Furthermore, activation of NK cells...
via 2B4 and NTB-A (9, 18–20), and activation of some signalling pathways downstream of SLAM in T cells (15, 16), requires the expression of functional SAP. Thus, XLP may result from an inability to signal through these receptors. Although the Y54C, F87S and I84T missense mutations we identified greatly reduced the level of SAP detected in MNCs, they did not completely abolish SAP expression (Figs 2b and 3, right panels). Thus, it remained possible that these mutated SAPs may still be capable of associating with the SLAM family of cell-surface receptors.

To test this, BaF/3 cell lines were generated that expressed either CD84, 2B4 or SLAM alone or co-expressed WT SAP or mutant Y54C, F87S or I84T SAP. The fs82 → X103 mutation was not tested in this experiment because it completely eliminated SAP expression (see Figs 1 and 2). The amounts of the surface receptor, as determined by flow cytometry and western blotting, were comparable for the different transductants expressing either WT or mutant SAP (data not shown; 10, 14). Similarly, the amounts of eGFP detected in the different SAP transductants were comparable (Fig. 2a). For these experiments, the CD84 and 2B4 transductants were first treated with sodium pervanadate to induce tyrosine phosphorylation of these proteins, a prerequisite for the binding of SAP (7, 8, 14). The cells were lysed and then immunoprecipitated with anti-SAP antiserum or a control antiserum and the resulting immunoprecipitates analysed for the presence of specific proteins and phosphoproteins. When lysates from cells expressing the receptors alone were incubated with anti-SAP antibody, no associating proteins recognized by anti-phosphotyrosine (Fig. 3a and b, upper panel, lanes 1 and 2) or anti-CD84 mAb were detected (Fig. 3a, middle panel, lanes 1 and 2). As expected, proteins corresponding to phospho-2B4, phospho-CD84 and CD84 were co-immunoprecipitated by anti-SAP, but not control rabbit Ig, from cell lines expressing WT SAP (Fig. 3a and b, lanes 3 and 4). The F87S and Y54C mutations abrogated binding of SAP to 2B4 (Fig. 3b, lanes 5–8). Binding to CD84 was completely disrupted by Y54C (Fig. 3a, lanes 7–8), yet only partially reduced by F87S (Fig. 3a, lanes 5–6). Interestingly, although the I84T mutation reduced the expression of SAP to a level similar to that of the other mutations (see Fig. 2b and d), this version of SAP retained the ability to bind 2B4 and CD84 (Fig. 3a and b, lanes 9 and 10). Similar experiments were performed using BaF/3 cells transduced with SLAM and either WT or mutated SAP; in these experiments, SLAM was immunoprecipitated and then assessed for association with SAP. Analogous to 2B4, SLAM failed to bind to the F87S and Y54C SAP mutants (Fig. 3c, lanes 3–6); however, binding to the I84T mutant was as efficient as binding to WT SAP (Fig. 3c, compare lanes 2 and 8). The differential effect of the distinct mutations on the expression of SAP and its ability to bind to defined SLAM family receptors in transfectants are summarized in Table 1.

These findings were extended by examining the ability of endogenous WT and mutant SAPs to associate with SLAM family receptors expressed by activated lymphocytes. PBMCs from a normal donor (WT) and three different XLP patients (XLP#1, 3 and 5) were expanded in vitro and then treated with sodium pervanadate to induce tyrosine phosphorylation. Endogenous SAP was then precipitated from the cells and the resulting immune complexes were assessed for the presence of SAP and associated phosphoproteins. Comparable amounts of SAP were immunoprecipitated from activated PBMCs from the normal donor as well as from XLP#1 and 3, which harbour the Y54C and F87S mutation, respectively (Fig. 4d, lower panel, lanes 1–6). In contrast, very little I84T SAP could be immunoprecipitated from lysates of activated PBMCs from XLP#5 (Fig. 4d, lower panel, lanes 7–8), suggesting the half-life of this mutant SAP is even less than that of Y54C and F87S SAP (Fig. 3). Immunoprecipitation of SAP from pervanadate-treated PHA blasts expressing WT SAP resulted in the detection of phosphoproteins with a molecular weight of ~65–80 kDa (Fig. 4d, upper panel, lane 2). Although the identity of these proteins was not individually determined, they are likely to correspond to 2B4, CD84 (both ~80–85 kDa) (7, 14, 44), SLAM (~70 kDa) (30) and/or NTB-A (65 kDa) (9), all of which are expressed on PHA-activated human T cells (9, 14, 30). When mutant SAPs were immunoprecipitated from XLP PHA blasts, interactions between F87S SAP and phosphoproteins were barely detectable (Fig. 4d, upper panel, lane 4), Y54C SAP exhibited some weak binding to such proteins; however, the level of interaction was greatly reduced compared with WT SAP (Fig. 4d, upper panel, compare lanes 2 and 6). Based on the data derived from examining BaF/3 transfectants expressing SLAM family receptors and the different mutant SAPs (Fig. 4a–c), it is likely that the phosphoprotein noted to associate with endogenous F87S is CD84, while that binding to Y54C may be NTB-A. On the other hand, I84T SAP failed to yield any detectable associating phosphoproteins (Fig. 4d, upper panel, compare lane 8). Although the data in Fig. 4(a–c) would have predicted a detectable association between I84T SAP and SLAM family receptors, it is likely that the inability to observe such an interaction is due to their being a greatly reduced amount of endogenous I84T SAP available in this experiment using primary cells (i.e. ~10-fold less than WT SAP; Fig. 4d, lower panel), compared with stable transfectants that have enforced expression of high levels of this protein (Fig. 4a, b, lower panels). Because of this, it cannot be discounted that I84T SAP is capable of binding SLAM family receptors in MNCs. Thus, different mutations in SAP may contribute to the development of XLP via numerous mechanisms, including diminishing protein expression as well as disrupting binding to members of the SLAM family of surface receptors.

**Table 1. Differential effects of mutations on expression and function of SAP**

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<tr>
<th>SAP</th>
<th>Expression</th>
<th>Binding</th>
<th>Activate 2B4</th>
<th>2B4</th>
<th>CD84</th>
<th>SLAM</th>
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The 2B4 function was assessed by the ability of NK cells from normal donors and XLP patients to be activated following ligation of 2B4 with a specific mAb, pSLAM, phosphorylated SLAM; n.d., not done.
Fig. 4. Missense mutations differentially affect the ability of SAP to bind to members of the SLAM family of surface receptors. Expression plasmids encoding WT or mutant SAP were transduced into BaF/3 cells already expressing (a) CD84, (b) 2B4 or (c) SLAM. The cells were treated with sodium pervanadate for 5 min at 37°C, solubilized and then immunoprecipitated with (a and b) control rabbit Ig (Rig) or anti-SAP antibody or (c) M2 anti-FLAG mAb (to capture FLAG-tagged SLAM). Precipitated proteins were electrophoresed through 15% polyacrylamide gels and transferred to PVDF membranes. The membranes were analysed for the presence of proteins phosphorylated on tyrosines (anti-pTyr, a–c), CD84 (a) or SAP (a–c). Untransduced BaF/3 cells (Nil) were used as a negative control. p, phosphorylated. Note: the protein below 2B4 is the heavy chain of the precipitating rabbit Ig that cross-reacts with the detecting reagent used for these blots. (d) PHA-blasts were generated from PBMCs isolated from a normal donor (lanes 1 and 2), XLP#3 (F87S, lanes 3 and 4), XLP#1 (Y54C, lanes 5 and 6) and XLP#5 (I84T, lanes 7 and 8) by stimulation with PHA and IL-2. After 10–14 days, the cells were collected, washed and then either untreated (–) or treated (+) with sodium pervanadate for 5 min at 37°C, solubilized and then immunoprecipitated with anti-SAP antibody. Precipitated proteins were electrophoresed through 15% polyacrylamide gels and transferred to PVDF membranes, which were analysed for the presence of proteins phosphorylated on tyrosines (upper panel) or SAP (lower panel).
form (Fig. 5a, lower panel). To test this, 293T cells were transfected with increasing amounts of WT SAP plasmid (0–12 μg), together with Fyn and SLAM, and the amount of SLAM tyrosine phosphorylation was assessed. As the amount of WT SAP plasmid transfected into the cells was increased, the extent of both SLAM phosphorylation (Fig. 5b, upper panel, lanes 2–6) and recruitment of SAP to SLAM (Fig. 5b, lower panel, lanes 2–6) incrementally increased. Even the lowest amount of WT SAP plasmid (1.5 μg) tested induced a significant level of SLAM tyrosine phosphorylation (Fig. 5b, compare lanes 2 and 3). When 293T cells were transfected with I84T SAP at a dose corresponding to the highest amount of WT SAP plasmid (i.e. 12 μg), together with Fyn and SLAM, the amount of SLAM tyrosine phosphorylation approximated that observed in cells transfected with SLAM, Fyn and the lowest amount of WT SAP tested (1.5 μg; Fig. 5b, upper panel, compare lanes 3 and 7). In this experiment, the amount of I84T SAP recruited to SLAM was comparable to the intermediate amounts of WT SAP (3–6 μg) that were capable of mediating tyrosine phosphorylation of SLAM (Fig. 5b, lower panel). Thus, despite retaining the ability to associate with SLAM-related receptors, the ability of mutant I84T SAP to induce downstream signalling through SLAM is ~4-fold less that that of WT SAP. This most likely results from its reduced ability to facilitate protein tyrosine kinase-mediated phosphorylation of SLAM, rather than reduced expression of I84T SAP. The other mutations detected were not examined in this experiment because they either ameliorated the recruitment of SAP to SLAM receptors (Y54C and F87S) or completely abolished SAP expression (fs82→X103; see Table 1).

Functional consequences of destabilizing SAP mutations

SAP is required for 2B4- and NTB-A-mediated activation of human NK cells (9, 18–20). Most patients examined previously had large genomic deletions of the SH2D1A gene or nonsense mutations that introduced premature stop codons, such that transcription of SH2D1A and the expression of SAP were completely abrogated. Based on our observation that mutant SAP I84T could still bind to SLAM receptors (Fig. 4), yet was impaired in its ability to activate Fyn (Fig. 6), it was of interest to test our above hypothesis that mutant I84T SAP would be unable to induce 2B4-mediated effector function. To investigate this, we assessed the ability of NK cells from XLP patients harbouring distinct missense mutations in SH2D1A to be activated through 2B4 in a redirected killing assay using the mouse mastocytoma cell line P815 as the target cell (18, 29). Cytolytic activity of normal NK cells was enhanced in the presence of anti-2B4 mAbs, and the level of augmentation was similar to that observed with anti-CD16 mAb (Fig. 6). In contrast, NK cells obtained from three unrelated XLP patients failed to exhibit heightened cytotoxicity following ligation of 2B4 (Fig. 6). However, these NK cells were responsive to

![Fig. 5. Mutant SAP I84T fails to facilitate tyrosine phosphorylation of SLAM. (a and b) The 293T cells were untransfected or transiently transfected with plasmids encoding SLAM, Fyn and/or WT or mutant I84T SAP. The cells were collected after 48 h and solubilized in 1% NP-40 buffer, and lysates were immunoprecipitated with M2 anti-FLAG mAb. Precipitated proteins were electrophoresed through 12% polyacrylamide gels and transferred to PVDF membranes. The membranes were analysed for the presence of phospho-SLAM (pSLAM, anti-pTyr blot, upper panel), SLAM (anti-FLAG, middle panel) and SAP (anti-SAP, lower panel). Similar results were obtained in three independent experiments. For (b), 293T cells were transfected with increasing amounts (0–12 μg) of plasmids encoding WT SAP or 12 μg I84T SAP only, together with plasmids encoding SLAM and Fyn, and analysed as above. Immunoblotting for Fyn revealed similar amounts of protein expression in all transfections (data not shown).](image)

![Fig. 6. Different missense mutations in SH2D1A abolish the ability of 2B4 to induce cytotoxicity of human NK cells. NK cells were purified from the peripheral blood of normal healthy donor (Ctl#1 and 2) or the indicated XLP patients with different mutations in SH2D1A, and then were cultured overnight with IL-2. The following day, the cells were assessed for cytotoxicity against P815 target cells in the presence of an isotype control mAb (clg) or mAb specific for 2B4 (c1.7) or CD16. Cytotoxicity was assessed using a europium-based lysis assay. Results are expressed as the percent lysis of target cells. Each value represents the mean of duplicate or triplicate cultures.)](image)
stimulatory signals, as evidenced by efficient anti-CD16 mAb-induced cytotoxicity (Fig. 6). The reduced level of killing by XLP#7 NK cells probably reflects the fact that this population contained only ~50% CD56+ cells (data not shown). Thus, irrespective of the type of mutation in SH2D1A, the outcome is impaired cytotoxicity delivered through the SAP-associating receptor 2B4 (see Table 1).

Discussion

In this current study, we examined four families affected by XLP and characterized the mutations detected in SH2D1A. Three missense mutations were identified, two of which have been reported previously (Y54C and F87S; 14, 21). However, a novel (I84T) missense mutation was detected in six members of one family. An XLP patient in the fourth family harboured an insertion/frameshift mutation that was also novel. A striking finding was that all of these SH2D1A mutations dramatically reduced the expression of SAP in primary lymphoid cells, as well as in stably and transiently transfected cell lines (Figs 2 and 3, Table 1). In previous studies that collectively examined 13 missense mutations different from those we identified, eight reduced the expression of SAP in transfectants by shortening its half-life (24, 42, 43). We extended this finding by demonstrating that the Y54C, F87S and I84T mutations had a similar effect on the half-life, and therefore stability, of SAP. This most likely results from gross changes that occur to the tertiary structure of SAP due to these amino acid replacements. Based on the crystal structure of SAP (12), several predictions can be made regarding how the mutations we identified would destabilize SAP. Because the Y54C mutation represents a change from a large cyclic amino acid to a small polar residue, it is near this region, that is proposed to be involved in forming contact sites with the TxY281xxV motif present in the cytoplasmic domain of SLAM (12, 37). The findings that F87S SAP could still bind phospho-CD84, but neither SLAM nor 2B4, and that Y54C may bind a SLAM family member other than those examined here are reminiscent of a previous study reporting that SAP containing a T53I mutation was unable to bind unphosphorylated SLAM and phospho-CD84 and Ly9, but did associate weakly with phospho-CD84 and normally with phospho-SLAM (24, 26). Thus, the requirements for binding of SAP to its associated receptors, as well as the actual dynamics of this process, may differ depending on the cell-surface receptor involved. In contrast to these mutations, SAP I84T was capable of binding to 2B4, CD84 and SLAM when over-expressed in transfected BaF/3 cells, including both the unphosphorylated and phosphorylated forms of SLAM, thereby demonstrating that this residue does not participate in the SLAM–SAP interaction. When compared with other SAP mutations that have been characterized biochemically (19 in total, including the four reported here), the I84T mutation appears to be unusual because only one other mutant (P101L) has been identified that has preserved binding to SLAM family receptors (24–26, 28, 43). This is consistent with the positioning of I84 and P101 distal to the regions required for the binding of SAP to SLAM (12, 37). Indeed, the affinity of P101L SAP for SLAM was reduced <2-fold, whereas that of most other mutants was reduced by ~10 to >100-fold (26, 28).

Despite I84T SAP being able to bind SLAM and related receptors, this appeared to be insufficient to facilitate downstream signalling processes to the same extent as WT SAP, as evidenced by its reduced ability to induce tyrosine phosphorylation of SLAM. Although our in vitro assay was not adequately sensitive to visualize the recruitment of Fyn to the SLAM–WT SAP complex (data not shown), it is likely that the reduced phosphorylation of SLAM by Fyn in the presence of SAP I84T resulted from sub-optimal recruitment of Fyn, rather than from reduced expression of I84T SAP. This is based on two observations. First, amino acid residues at position 75–82 are involved in the binding of SAP to Fyn (16). Because I84T is near this region, this mutation may compromise the ability of SAP to associate with Fyn. Second, even low microgram quantities of WT SAP plasmid, which approximated the level of expression of I84T SAP, were sufficient to induce the phosphorylation of SLAM. This would make it unlikely that reduced SLAM phosphorylation in the presence of I84T SAP resulted from reduced expression of I84T SAP. Based on our observation that the Y54C and F87S SAP mutants generally did not bind SLAM family receptors, we did not examine directly whether these mutants were capable of binding to and activating Fyn. However, a recent study addressed this issue, and, similar to reducing the affinity of SAP for SLAM, it was found that the affinity of these mutant SAPs for Fyn was 10-fold less than that of WT SAP (28). Based on our results, it is likely that the affinity of mutant SAP for CD84 and 2B4 is reduced to a similar extent. The ability of the Y54C and F87S mutations to interfere with SAP binding is consistent with these residues being located within regions of the βD-sheet and αB-helix of SAP, respectively, that are proposed to be involved in forming contact sites with the TxY281xxV motif present in the cytoplasmic domain of SLAM (12, 37). These results are consistent with a recent study that reported that the affinity of some SAP mutants for SLAM was reduced >25-fold compared with that of WT SAP (28). Based on our results, it is likely that the affinity of mutant SAP for CD84 and 2B4 is reduced to a similar extent. The ability of the Y54C and F87S mutations to interfere with SAP binding is consistent with these residues being located within regions of the βD-sheet and αB-helix of SAP, respectively, that are proposed to be involved in forming contact sites with the TxY281xxV motif present in the cytoplasmic domain of SLAM (12, 37). These results are consistent with a recent study that reported that the affinity of some SAP mutants for SLAM was reduced >25-fold compared with that of WT SAP (28). Based on our results, it is likely that the affinity of mutant SAP for CD84 and 2B4 is reduced to a similar extent. The ability of the Y54C and F87S mutations to interfere with SAP binding is consistent with these residues being located within regions of the βD-sheet and αB-helix of SAP, respectively, that are proposed to be involved in forming contact sites with the TxY281xxV motif present in the cytoplasmic domain of SLAM (12, 37).
less than that of WT SAP (28). Taken together, signalling downstream of SAP-associating receptors would be blocked in lymphocytes from XLP patients bearing the mutations examined here because SAP would be impaired in its ability to bind to SLAM-related receptors, Fyn or both. Indeed, NK cells from all XLP patients examined exhibited a selective impairment in 2B4-mediated activation of their cytotoxic effector function irrespective of the type of missense mutation, similar to previous observations when XLP patients with large genomic deletions in their SH2D1A gene were examined (18, 19). Altogether, these findings indicate that signalling through SAP-associating receptors in XLP will be severely impaired through one or more simultaneously acting mechanisms—(i) reducing the half-life SAP, thereby diminishing its expression; (ii) reduced binding of SAP to SLAM family receptors and (iii) reduced ability to activate signal transduction downstream of the SLAM–SAP complex.

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Abbreviations

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<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>IRES</td>
<td>internal ribosome entry site</td>
</tr>
<tr>
<td>MNC</td>
<td>mononuclear cell</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>SA</td>
<td>streptavidin</td>
</tr>
<tr>
<td>SAP</td>
<td>signalling lymphocytic activation molecule-associated protein</td>
</tr>
<tr>
<td>SH2</td>
<td>src-homology-2</td>
</tr>
<tr>
<td>SLAM</td>
<td>signalling lymphocytic activation molecule</td>
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<td>XLP</td>
<td>X-linked lymphoproliferative disease</td>
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<tr>
<td>WT</td>
<td>wild-type</td>
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