The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM

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In addition to triggering the activation of B- or T-cell antigen receptors, the binding of a ligand to its receptor at the cell surface can sometimes determine the physiological outcome of interactions between antigen-presenting cells, T and B lymphocytes. The protein SLAM (also known as CDw150), which is present on the surface of B and T cells, forms such a receptor–ligand pair as it is a self-ligand. We now show that a T-cell-specific, SLAM-associated protein (SAP), which contains an SH2 domain and a short tail, acts as an inhibitor by blocking recruitment of the SH2-domain-containing signal-transduction molecule SHP-2 to a docking site in the SLAM cytoplasmic region. The gene encoding SAP maps to the same area of the X chromosome as the locus for X-linked lymphoproliferative disease (*XLP*) and we found mutations in the SAP gene in three XLP patients. Absence of the inhibitor SAP in XLP patients affects T/B-cell interactions induced by SLAM, leading to an inability to control B-cell proliferation caused by Epstein–Barr virus infections.

SLAM (for signalling lymphocyte-activation molecule), is a glycosylated type-I transmembrane protein, of relative molecular mass 70K, that is present on the surface of B and T cells; it is a high-affinity self-ligand. As triggering of SLAM co-activates T- or B-lymphocyte responses, it is considered to be important in bidirectional T ↔ B-cell stimulation[1–7]. Monoclonal antibodies directed against SLAM induce proliferation of human $CD45RO^{high}$ memory T cells in a CD28-independent manner, whereas resting T cells are unresponsive[1–3]. Anti-SLAM acts as a co-activator, together with antibodies against the T-cell antigen receptor (TCR) or complexes of peptides with the major histocompatibility complex (MHC) on the surface of antigen-presenting cells, in stimulating resting T cells to synthesize DNA and/or to initiate cytokine gene activation cascades[1,2]. Triggering of signals by anti-SLAM antibodies induces the interferon-γ gene and redirects Th2 responses of antigen-specific T-cell clones to a Th1 or Th0 phenotype[3–5].

Exposure of B cells to a soluble form of the SLAM ectodomain or to L cells transfected with membrane-bound SLAM dramatically enhances B-cell proliferation and production of IgM, IgG and IgA, in conjunction with anti-CD40 monoclonal antibodies or other co-stimuli[6]. Anti-SLAM antibodies alone fail to enhance B-cell proliferation, even having an inhibitory effect[6]. Thus, SLAM-induced signal-transduction events in T lymphocytes are different from those in B cells.

Cloning of SAP

In order to determine the mechanism by which SLAM induces signalling, we identified proteins that interact with the cytoplasmic domain of human SLAM. Using a yeast two-hybrid system with the 77-amino-acid human SLAM cytoplasmic domain as bait and a human T-cell library, eight complementary DNA clones were isolated that encoded a 128-amino-acid polypeptide chain which we term SLAM-associated protein, or SAP. To confirm that the cDNAs were full-length, we also isolated cDNA clones from a human T-cell (Jurkat) library and from a murine (C57B16) thymus library. The predicted human SAP protein comprises a single SH2 domain (residues 6–102) followed by a short 26-amino-acid tail (residues 103–128). Human and murine SAP are highly homologous (96%) in both the SH2 and tail domains (Fig. 1). The SAP SH2 domain was clearly related to other SH2 domains, particularly those of SHIP (45%), EAT-2 (40%) and Abl (35%), but its short tail was unlike any known protein domain.

Antibodies directed against human or mouse SAP-tail sequences detected a 15K protein in detergent lysates made from either human T cells (Fig. 2a) or murine thymocytes (Fig. 2b). Because the observed relative molecular mass was consistent with the predicted value, our human and mouse SAP cDNAs are unlikely to code for a fragment of a larger protein. We found that SLAM and SAP interact in T lymphocytes because a human SAP fusion protein, with glutathione-*S*-transferase, can specifically precipitate SLAM from detergent lysates made from a murine T-cell line (EL-4) transfected with human SLAM. By contrast, no interaction was detected in lysates made with untransfected EL-4 cells (Fig. 2c). Conversely, SAP was co-immunoprecipitated with anti-SLAM antibodies using detergent lysates made from activated human peripheral blood lymphocytes[5] (Fig. 2d).

Identification of SAP as a T-cell protein

Human SAP messenger RNA expression was highest in the thymus and less in spleen and peripheral blood lymphocytes (Fig. 3a). SAP was expressed in all major subsets of human T cells ($CD4^+$, $CD45RO^+$, $CD45RA^+$ and $CD8^+$) (Fig. 3c), in the T-cell tumour cell line Jurkat, and in the Burkitt lymphoma line Raji which is positive in the Epstein–Barr virus (EBV). No transcripts were detected in the EBV^+ Burkitt lymphomas Namalwa or BL41/B95-8, the EBV-transformed cell line X50-7, the EBV^+ marmoset cell lines B95-8 and FF41, the EBV^- Burkitt lymphoma BJAB, or in the pre-B ALL lines LAZ 221 and NALM 6 (Fig. 3, and data not shown).

Figure 1 Comparison of deduced amino-acid sequences of human SAP, murine SAP and other SH2-domain-containing proteins. Amino acids that are only found in human and murine SAP are in blue; amino acids found in other SH2 domains are in red. Consensus α-helices and β-sheets are indicated in brackets. Exon/intron boundaries as derived from the murine SAP gene (C.W., J.S. and C.T., manuscript in preparation) are demarcated by downward arrows. Four truncation mutants were generated in the tail of human SAP (del 1–4) and their locations in the amino-acid sequence are shown (upward arrows). Comparisons are made between SH2 domains that were found to be most similar in a computer-aided search, namely human SHIP, murine EAT-2 and human Abl. A computer-aided search for the SAP cDNA in the EST library identified three human clones (accession numbers N89899, W19453 (fetal lung) and AA354319 (Jurkat)) and one highly homologous murine T-cell-derived clone of 294 bp (accession number AA255258).

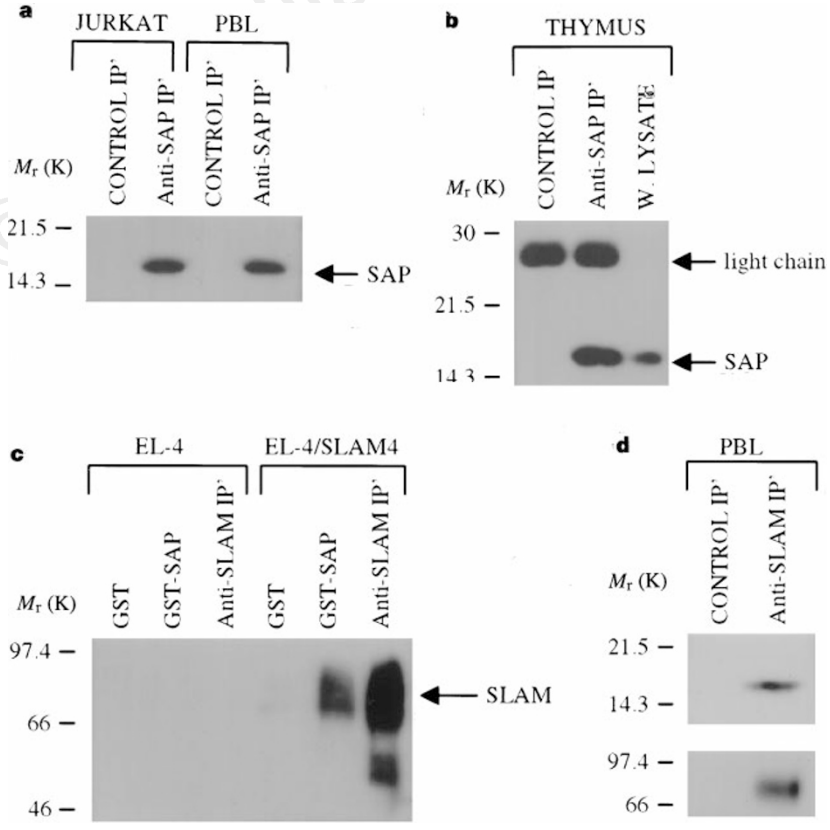

Figure 2 Characterization of the human and murine SAP proteins and their interactions with SLAM. **a**, Jurkat cells and human peripheral blood lymphocytes (PBL) were lysed and postnuclear lysates (1 mg ml^{-1}) were immunoprecipitated (IP) with a rabbit antiserum directed against human SAP or with a control serum (3 μl). Samples were analysed on SDS–PAGE and immunoblotted with anti-human-SAP (1/1,000). **b**, Postnuclear lysates (1 mg ml^{-1}) from C57B1/6 thymocytes were immunoprecipitated with an anti-murine SAP antibody (3 μl), resolved on SDS–PAGE and immunoblotted with anti-mouse-SAP (1/1,000). **c**, SLAM- or vector-transfected EL-4 cells were cell-surface biotinylated and lysed in detergent. Postnuclear lysates (1 mg ml^{-1}) were incubated with 5 μg GST or GST–SAP for 1 h in the presence of glutathione beads or 1 μg anti-SLAM antibodies (a gift from DNAX Research Institute) for 3 h in the presence of protein G beads. Bead-associated proteins were extracted and immunoblotted with streptavidin–HRP. **d**, Human PBL were activated with phytohaemagglutinin for 5 d. Cells were lysed (1×10^8 per ml) and immunoprecipitated with an anti-SLAM monoclonal antibody or with a control antibody. Immunoprecipitates were resolved on SDS–PAGE and immunoblotted with anti-SLAM serum (top) or with a rabbit anti-SAP antibody (bottom).

A small amount of SAP mRNA detected in the small intestine probably resulted from lymphocyte contamination. In wild-type mice, SAP is expressed in T cells but not in B cells, whereas no mRNA or protein could be detected in CD3ε^{null} mice which lack T cells (C.W., J.S. and C.T., manuscript in preparation). Both human mRNA species encoding SAP (2.5 and 0.9 kilobases (kb)) (Fig. 3) were represented among the cDNA clones isolated in the two-hybrid system and encoded the same open reading frame, but differed in their 3′ untranslated sequences. Collectively, our results show that, unlike SLAM, SAP is primarily expressed in T cells.

SAP is encoded by the XLP disease gene

By using a 45-kb pBAC clone that contained all four exons of murine SAP (Fig. 1), the SAP gene was localized within band A5.1 of the murine X chromosome (C.W., J.S. and C.T., manuscript in preparation). Because of synteny between murine band A5.1 and human Xq25, where the locus for the immune deficiency X-linked lymphoproliferative disease (XLP) had been mapped[8–11], we investigated whether the SAP gene could be involved in this disease. Moreover, the observation that in XLP uncontrolled B-cell proliferation following EBV infection often leads to fatal infectious mononucleosis, malignant lymphomas, hypogammaglobulinaemia or aplastic anaemia[11,12] was in agreement with the involvement of SLAM in T ↔ B-cell interactions. To test the idea that SAP is encoded by the XLP gene, we isolated SAP cDNAs from peripheral blood mononuclear cells of three XLP patients: a previously described patient, A1 (ref. 13), and two brothers, B1 and B2, who were recently diagnosed as having XLP.

XLP patient A1 developed hypogammaglobulinaemia and recurrent pulmonary infections a few months after severe EBV-induced infectious mononucleosis[13]. His family history and the persistence of an unbalanced virus–host relationship after EBV infection are consistent with the main features of XLP syndrome[13]. When the products from reverse transcription with polymerase chain reaction (RT-PCR) of T cells from patient A1 were analysed by polyacrylamide gel electrophoresis (Fig. 4a), three products were initially found of 629 base pairs (bp) (the full-length SAP coding sequence), 565 bp and 520 bp. On cloning the RT-PCR products of patient A1, four different mRNAs were identified: namely, full-length hSAP; a coding sequence, ΔE2, which lacked the 64 nucleotides of exon 2 (A1-1 in Fig. 5); E3Δ55, with a 55-nucleotide deletion in exon 3 (hSAPΔ55 in Fig. 5); and a cDNA with both a deleted exon 2 and the deletion E3Δ55 (Al-2 in Fig. 5). T cells from patient A1 thus had four mRNA species coding for different forms of SAP, of which two were detected in healthy individuals (629 and 574 bp) (Fig. 5) and two were specific for the patient (A1-1 of 565 bp and A1-2 of 520 bp; note that the 574- and 565-bp species cannot be separated by PAGE in Fig. 4). The predicted amino-acid sequences were indicative of two truncated proteins, in which essentially only the first exon of SAP is intact.

RT-PCR products from peripheral blood mononuclear cells of 60

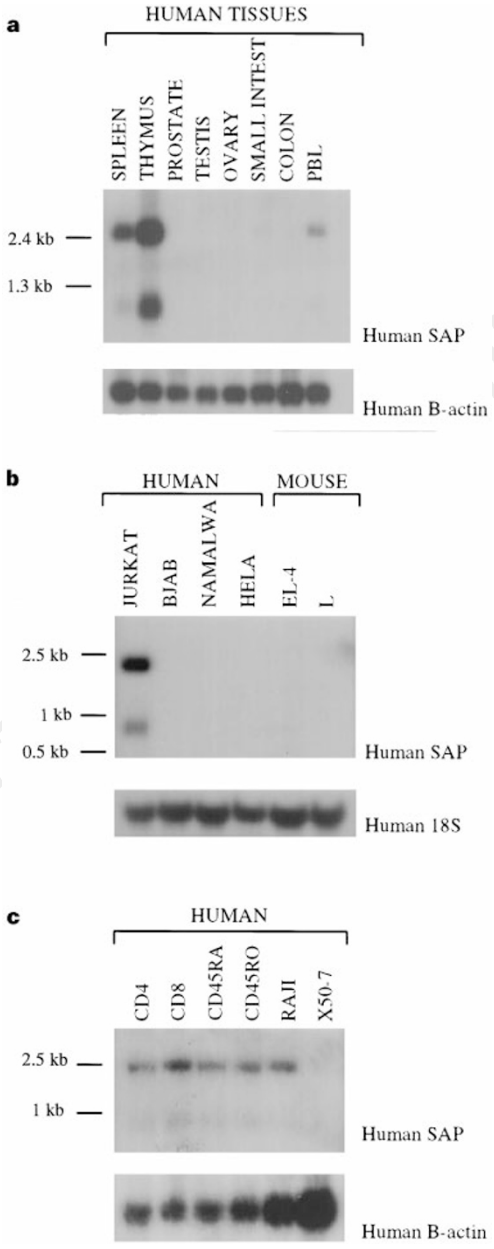

Figure 3 SAP expression. Northern blot analysis of **a**, poly(A)$^+$ mRNAs isolated from various human tissues (Clontech); **b**, total RNAs from different human and mouse cell lines (20 μg per lane); or **c**, human T-cell subsets and two EBV$^+$ B-cell lines. Specific RNAs were detected using a ^{32}P-radiolabelled human SAP probe; β-actin or 18S rRNA probes were used as loading controls.

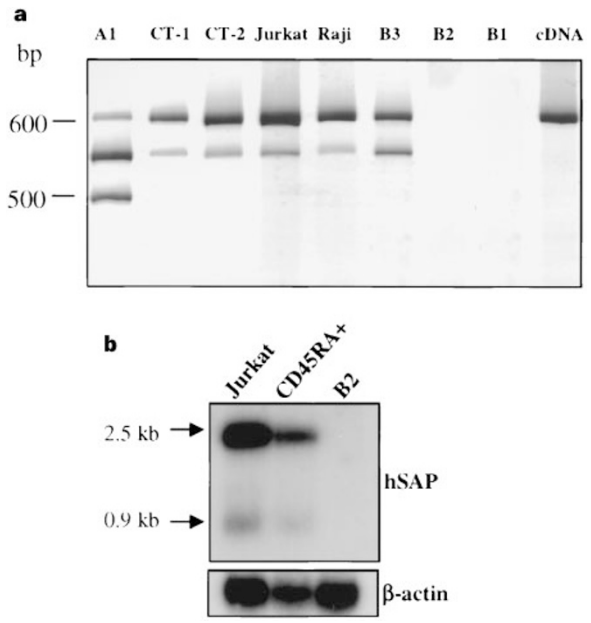

Figure 4 mRNA analysis of XLP patients. **a**, Complementary DNA was isolated from peripheral blood mononucleocytes (PBMC) from patients and healthy donors by RT-PCR. DNA products were resolved on 10% polyacrylamide gels. Samples A1, B1 and B2 are from XLP patients; sample B3 is from a healthy brother of B1 and B2; samples CT-1 and CT-2 are from healthy controls. **b**, Northern blot analysis of total RNAs from Jurkat T cells, from a subset of CD45RA^{high} cells from a healthy donor, and from PBMC from patient B2 (20 μg per lane). Specific RNAs were detected using a ^{32}P-radiolabelled human SAP probe; a β-actin probe was used as loading control.

healthy individuals contained two mRNA species (of 629 and 574 bp), as judged by PAGE and nucleotide-sequence analysis (Figs 4a, 5). These represented hSAP and E3Δ55 (hSAPΔ55 in Fig. 5a) in which part of exon 3 had been deleted. The E3Δ55 coding sequence started at the beginning of exon 3 at nucleotide position 288 and ended at nucleotide 342 (Fig. 5a). Because of a frameshift, the predicted protein sequence was identical to that of the first two exons of SAP (amino acids 1–67), followed by a nine-amino-acid sequence and a stop codon (Fig. 5b). The results taken together indicate that the mRNAs of patient A1 had a deleted exon 2 or a deletion in exon 2 plus part of exon 3. These mutant forms derived from patient A1 were estimated to represent 90% of his SAP mRNA.

The 55-nucleotide deletion in exon 3 in healthy individuals is readily explained by the presence of an infrequently used splicing acceptor site within that exon: CAG preceded by a CT-rich stretch of nucleotides[14] (Fig. 6a). But to explain the frequent deletion of exon 2 in patient A1, the intron sequences surrounding exons 1, 2 and 3 had to be determined, so we designed a PCR assay based on sequences of at least 150 nucleotides of all four introns. This was facilitated by a new sequence in the EMBL database (accession number AL022718) indicating that *XLP* encoded a protein, SH2DIA. Its cDNA, genomic DNA and protein sequences were identical to human SAP, and in addition the exon/intron boundaries reported were identical to those in the mouse gene (Fig. 1). Upon sequencing the 443-bp PCR product derived from the exon-2 area, we found a C → G mutation in the nucleotide adjacent to the exon 2 splice acceptor site in the DNA of patient A1 (Fig. 6a). This mutation is known to affect the efficiency of the splice acceptor site[15] and explains the skipping of exon 2 in most (~90%) of the patient's SAP mRNA species.

To exclude the possibility that the point mutation found in XLP patient A1 represented a genetic polymorphism, we designed a restriction enzyme assay based on the fact that the C → G mutation generated a novel *Mnl*I restriction site in the 443-bp exon-2 PCR product. As shown in Fig. 6b, *Mnl*I generated four fragments (of 187, 151, 64 and 41 bp) with Al's 443-bp PCR product, whereas DNA samples from 108 healthy individuals generated three fragments (of 251, 151 and 41 bp) (see, for example, CT-1, CT-2 and B3 in Fig. 6b, and results not shown). Because patient A1 was Italian, these controls included DNA from 50 healthy Italian females (that is, 100 X chromosomes). The remaining 86 X chromosomes were from individuals with a random genetic background in the Boston area (28 female and 30 male). Collectively, our results demonstrate that A1 has a mutation affecting the exon 2 splice acceptor site, which gives rise to severely truncated forms of SAP. As this mutation was not detected in 186 X chromosomes from healthy individuals, it

a

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 87 ATGGACGCAGTGGCTGTGTATCATGGCAAAATCAGCAGGGAAACCGGCGAGAAGCTCCTGCTT   hSAP

150 GCCACTGGGCTGGATGGCAGCTATTTGCTGAGGGACAGCGAGACCGTGCCAGCCGTGTACTGC   hSAP

213 CTA TGT GTG CTG TAT CAC GGT TAC ATT TAT ACA TAC CGA GTG TCC CAG   hSAP
    CTA TGT GTG CTG TAT CAC GGT TAC ATT TAT ACA TAC CGA GTG TCC CAG   hSAPΔ55
    CTA TGT GTG CT- --- --- --- --- --- --- --- --- --- --- --- ---   A1-1
    CTA TGT GTG CT- --- --- --- --- --- --- --- --- --- --- --- ---   A1-2

261 ACA GAA ACA GGT TCT TGG AGT GCT GAG ACA GCA CCT GGG GTA CAT AAA   hSAP
    ACA GAA ACA GGT TCT TGG AGT GCT GAG --- --- --- --- --- --- ---   hSAPΔ55
    --- --- --- --- --- --- --- --- --- ACA GCA CCT GGG GTA CAT AAA   A1-1
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---   A1-2

309 AGA TAT TTC CGG AAA ATA AAA AAT CTC ATT TCA GCA TTT CAG AAG CCA   hSAP
    --- --- --- --- --- --- --- --- --- --- --- -CA TTT CAG AAG CCA   hSAPΔ55
    AGA TAT TTC CGG AAA ATA AAA AAT CTC ATT TCA GCA TTT CAG AAG CCA   A1-1
    --- --- --- --- --- --- --- --- --- --- --- -CA TTT CAG AAG CCA   A1-2

357 GATCAAGGCATTGTAATACCTCTGCAGTATCCAGTTGAGAAGAAGTCCTCAGCTAGAAGTACA   hSAP

420 CAAGGTACTACAGGGATAAGAGAAGATCCTGATGTCTGCCTGAAAGCCCCATGA           hSAP
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b

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                    EXON 1
 1 MDAVAVYHGKISRETGEKLLLATGLDGSYLLRDSESVPGVYCLCVL YHGYI   hSAP
   MDAVAVYHGKISRETGEKLLLATGLDGSYLLRDSESVPGVYCLCVL YHGYI   hSAPΔ55
   MDAVAVYHGKISRETGEKLLLATGLDGSYLLRDSESVPGVYCLCVL QHLGY   A1-1
   MDAVAVYHGKISRETGEKLLLATGLDGSYLLRDSESVPGVYCLCVL ISEAR   A1-2

     EXON 2                    EXON 3
   YTYRVSQTETGSWSAE TAPGVHKRYFRKIKNLISAFQKPDQGIVIPLQYPV   hSAP
   YTYRVSQTETGSWSAE HFRSQIKAL   76                         hSAPΔ55
   IKDISGK     58                                         A1-1
   SRHCNTSAVSS   62                                       A1-2

                    EXON 4
   EKKSSARSTQGTT GIREDPDVCLKAP  128                       hSAP
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Figure 5 Nucleotide and amino-acid sequences of SAP isolated from a patient with XLP. **a**, Comparison of the nucleotide sequence of two cDNAs isolated by RT-PCR from an XLP patient with those of human SAP (hSAP and hSAPΔ55). **b**, Comparison of the predicted amino-acid sequence of SAP from an XLP patient with that of normal human SAP. DNA products were cloned in the pCR2.1 vector and the nucleotide sequence determined from two cDNA clones in both directions in an ABI prism 377 DNA sequencer.

did not represent a genetic polymorphism. These results indicate that SAP is encoded by the XLP gene.

The two other XLP patients, brothers B1 and B2, had a deletion of the SAP gene, whereas a healthy brother, B3, had a normal SAP gene. B1 was a 23-year-old male with a history of recurrent pulmonary infections, dysgammaglobulinaemia (raised IgA and IgM), poor specific antibody responses to tetanus toxoid antigen, and a depressed T-cell proliferative response to mitogens. He developed fever, pneumonia and hilar adenopathy which quickly progressed to fulminant infectious mononucleosis with haemophagocytosis; EBV infection was documented by PCR and serology. B2 also suffers from XLP syndrome, which manifests as pancytopaenia, splenomegaly, dysgammaglobulinaemia and depressed T-cell proliferative responses to mitogens. B3 appears to be healthy, but two other brothers, for whom no material was available, had classic XLP symptoms: one has recurrent EBV^+ non-Hodgkin's lymphomas and another died with a diagnosis of Wegener's granulomatosis and pulmonary infiltration.

In contrast to the healthy sibling B3, no RT-PCR product encoding hSAP could be isolated from the peripheral blood mononuclear cells of patients B1 and B2 (Fig. 4a). This was because no mRNA was detected in northern blotting experiments with RNA from patients B1 (data not shown) and B2 (Fig. 4b). Thus, either the SAP gene was not transcribed in T cells from B1 and B2 or the gene was deleted, which commonly happens in XLP patients[11,12]. As none of the four exons encoding SAP could be generated in a PCR analysis based upon genomic DNA from B1 and B2 (Fig. 6c) and because a control PCR reaction for exon 2 of *BRCA1* gave the expected product, we conclude that the SAP gene was deleted in XLP patients B1 and B2. By contrast, DNA from B3 generated PCR fragments of the expected size and with the wild-type sequence (a similar result has been obtained by J. Sumegi, personal communication). These analyses of XLP patients A1, B1 and B2 show that the XLP gene encodes SAP.

Binding of the SH2 domain of SAP

Identification of SAP as the gene product that is altered in XLP raised the question of how this SLAM-associated T-cell protein could account for the immunological disturbances of the disease. The SLAM cytoplasmic domain contains three tyrosine residues (Y281, Y307 and Y327) that are surrounded by consensus SH2-domain-binding sequences[1], which suggests a possible interaction with the SH2 domain of SAP. Because SAP binds to SLAM in the yeast two-hybrid system, the binding of the SAP SH2 domain to SLAM must be more complex than the usual interactions with a short phosphotyrosine-containing peptide.

To determine to which portion of the cytoplasmic domain of SLAM the SAP SH2 domain binds, we co-expressed constructs encoding the CD8 extracellular and transmembrane domains and truncated cytoplasmic domains of SLAM with human SAP in COS-7 cells (Fig. 7a). Both CD8–SLAM proteins, that is CD8–SLAM3 (derived from a natural variant of SLAM with a short cytoplasmic domain containing only Y281) and CD8–SLAMdel1 (containing Y281 and Y307), co-precipitated SAP with the same efficiency as SLAM itself (Fig. 7a); two control chimaeric proteins, comprising the CD8 transmembrane and ecto domains and either the CD3-ϵ or the CD3-ζ cytoplasmic domain, did not co-precipitate SAP (Fig. 7a). This suggested the presence of a specific SAP-binding site around the most membrane-proximal tyrosine residue (Y281) of the SLAM cytoplasmic tail. As predicted from the *in vivo* results, only a peptide (SLAM Y1) containing Y281 coupled to agarose beads was able specifically to precipitate SAP from murine thymocyte lysates, and not peptides containing the other tyrosine residues in the cytoplasmic domain of SLAM (Fig. 7b). More important, binding of SAP to agarose-coupled SLAM Y1 was blocked only by the same peptide regardless of whether Y281 was replaced by phenylalanine (SLAM F1) or by a peptide in which phosphotyrosine replaced Y281 (SLAM pY1) (Fig. 7c). This confined the binding site to a short amino-acid sequence in SLAM and confirmed that

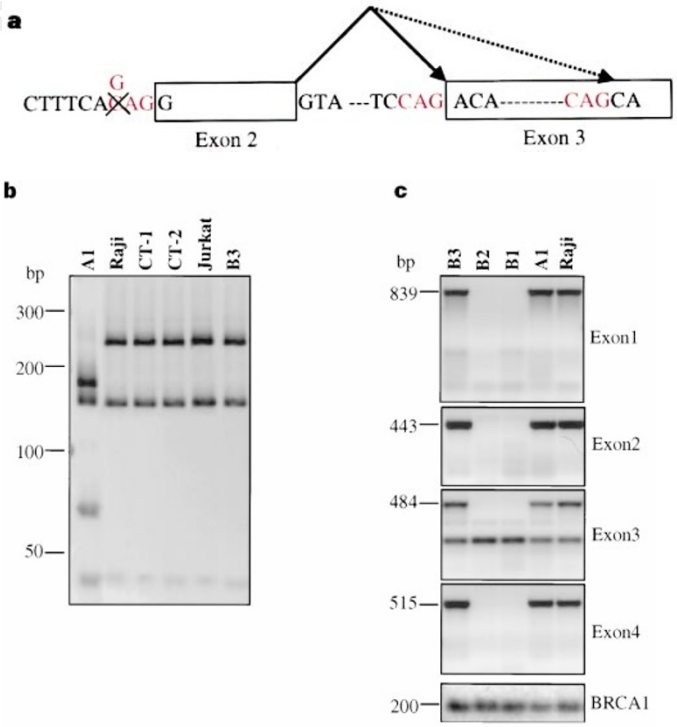

Figure 6 Genomic analysis of patients with XLP. **a**, Location of the point mutation near exon 2 of patient A1; a mechanism is shown to explain the generation of the variant from hSAPΔ55 found in all healthy individuals. **b**, Exon 2 sequence was amplified by PCR from genomic DNA from patient A1 and from three healthy individuals (CT-1, CT-2 and B3) and from two cell lines (Raji and Jurkat). In addition, PCR products from 78 healthy women and 30 healthy men were analysed (data not shown). DNA products were digested with *Mnl*I and resolved on a 10% polyacrylamide gel by standard methods. **c**, hSAP exon 1, exon 2, exon 3 and exon 4 and *BRCA1* exon 2 were amplified by PCR from genomic DNA from patients A1, B1, B2 and B3, and from the cell line Raji. DNA products were resolved on a 2% agarose gel.

phosphorylation of Y281 was not required for binding of the two polypeptide chains. However, we found that the SH2 domain of SAP was functionally intact as it could be purified on a Sepharose–phosphotyrosine column (data not shown).

In addition, four SAP mutants with deletions in the tail domain (Fig. 1) and co-transfected with the CD8–SLAM3 chimaera were able to interact with the short cytoplasmic domain of SLAM3, which contained the Y281 segment (Fig. 7d). Collectively these results show that SLAM interacts with SAP through part of its SH2 domain.

SAP blocks recruitment of SHP-2 to SLAM

The novelty of SLAM–SAP recognition, in which the usual requirements for SH2-domain interactions seem to be altered, suggested that SAP could block physiological reactions involving SLAM, acting as a natural inhibitor. This model would predict that SAP mutants that are unable to bind to SLAM could not act as inhibitors. We were able to test this hypothesis because we previously found (J.S. *et al.*, manuscript in preparation) that after phosphorylation by c-*fyn*, SLAM recruited the tyrosine phosphatase SHP-2.

First, two SAP mutants were tested: the A1 deletion (Δexon2 + E3Δ55) and a classic mutation in the SH2 domain that affects binding of phosphotyrosine (SAP32R → Q). Neither mutant bound to the cytoplasmic tail of SLAM, as judged by *in vivo* (Fig. 8a) or *in vitro* (Fig. 8b) assays. Thus, neither SAP mutant could act as a dominant-negative mutant through SLAM. More important, the experiment with mutant SAP32R → Q revealed that the interaction of SAP and SLAM depends on an intact phosphotyrosine binding pocket. An XLP patient with a mutation in R32 has recently been found (J. Sumegi, personal communication). These results, and the failure of the two SAP mutants to bind in patients, further support the idea that SAP–SLAM interaction is defective in XLP.

Next, SLAM and c-*fyn* were co-transfected with or without SAP into COS-7 cells, which contain endogenous SHP-2. If SLAM was phosphorylated by c-*fyn*, SHP-2 was co-precipitated with anti-SLAM antibody (Fig. 8c); in the absence of c-*fyn*, SLAM did not bind SHP-2. Introduction of SAP, together with SLAM and c-*fyn*, blocked the binding of SHP-2 and SLAM. The absence of SHP-2 in the presence of SAP could not be due to lesser phosphorylation of SLAM, because a significantly larger percentage of SLAM molecules was phosphorylated in the presence of SAP than in its absence. These results were confirmed by reciprocal co-immunoprecipitation with anti-SAP reagents (data not shown). By contrast (Fig. 8d), mutant SAP32R → Q did not prevent binding of SHP-2 to SLAM. A similar result was obtained with the A1 deletion mutant (data not

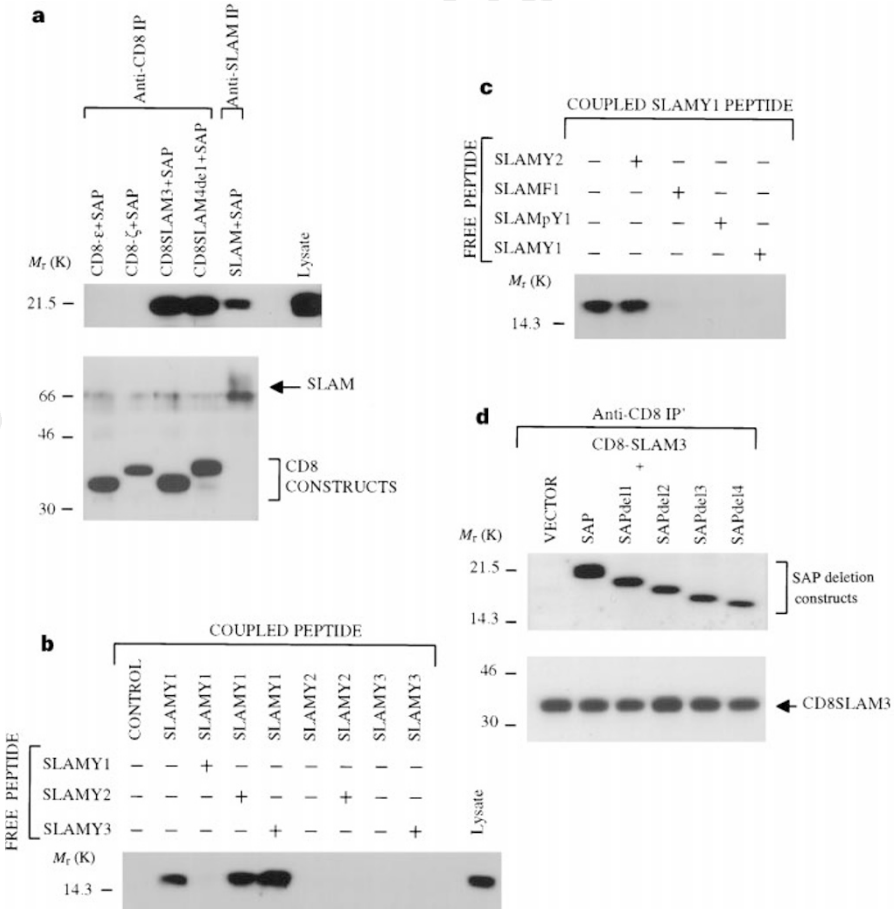

Figure 7 The SAP SH2 domain binds to a specific sequence in the cytoplasmic domain of SLAM. **a**, COS-7 cells were transfected, biotinylated and lysed. Postnuclear lysates were immunoprecipitated with antibodies anti-CD8 (OKT8) or anti-SLAM (ref. 1) and immunoprecipitates were immunoblotted with anti-human-SAP rabbit sera (top panel) or streptavidin (bottom). **b**, Postnuclear mouse thymocyte lysates (1 mg ml^{-1}) were incubated for 1 h with different peptides coupled to beads (7 μM) or with control beads in the absence or presence of free peptides (280 μM). Bead-associated proteins were extracted and immunoblotted with an anti-human-SAP rabbit serum (1/1,000). **c**, Postnuclear mouse thymocyte lysates (1 mg ml^{-1}) were incubated for 1 h with SLAM Y1 peptide coupled to beads (7 μM) in the absence or presence of free peptides (70 μM). Bead-associated proteins were extracted and immunoblotted with an anti-murine-SAP rabbit serum (1/1,000). **d**, COS-7 cells were co-transfected with CD8-SLAM3 construct and SAP-deletion mutant constructs del1, del2, del3 or del4 (Fig. 1). Cell-surface proteins were biotinylated and cells were lysed 48 h after transfection. Postnuclear lysates were immunoprecipitated with 1 μg anti-CD8 antibody (OKT8) and immunoprecipitates were immunoblotted with anti-Flag antibody (Kodak) (1/1,000) (top panel) or streptavidin (bottom panel).

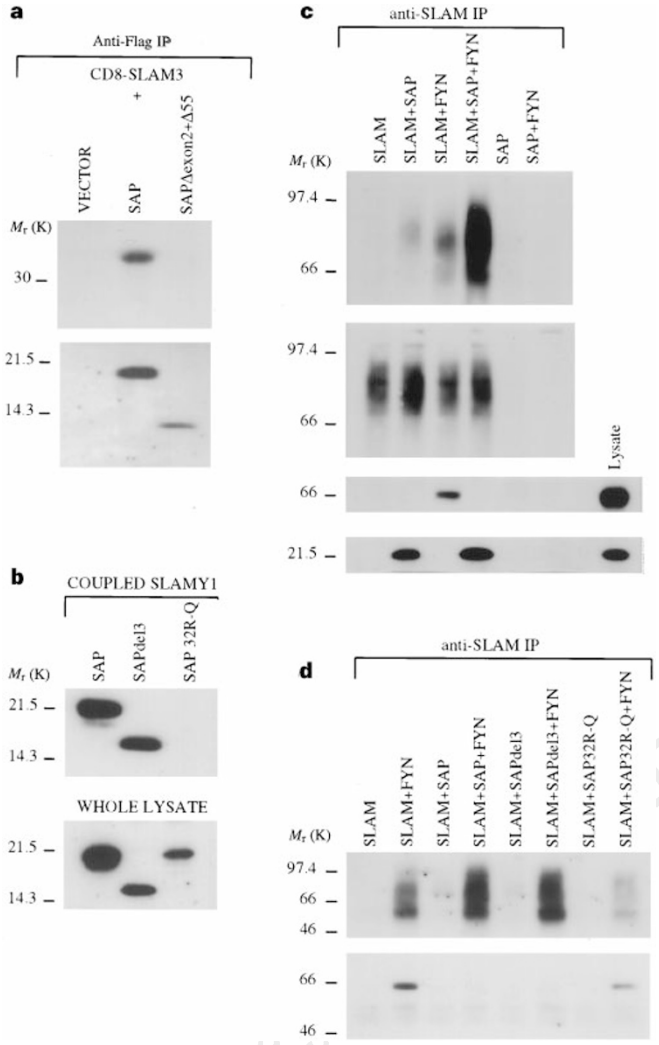

Figure 8 SAP blocks recruitment of the tyrosine phosphatase SHP-2 to the phosphorylated cytoplasmic domain of SLAM. COS-7 cells were transfected as indicated, cell-surface biotinylated and then lysed. Postnuclear lysates were **a**, immunoprecipitated with anti-Flag to isolate SAP and the associated CD8–SLAM3 chimaera; immunoprecipitates were immunoblotted with either streptavidin (upper panel) or anti-Flag (lower panel); or **b**, incubated for 1 h with the SLAM Y1 peptide coupled to beads (7 μM). Bead-associated proteins were extracted and immunoblotted with anti-Flag (1/1,000). **c**, **d**, COS-7 cells were co-transfected with a combination of SLAM, SAP and c-*fyn* constructs. 48 h after transfection, cell-surface-expressed proteins were biotinylated, and cells were lysed as described in Methods. Postnuclear lysates were immunoprecipitated with anti-SLAM antibodies and immunoprecipitates were immunoblotted with anti-phosphotyrosine–HRP (Zymed), streptavidin–HRP (Zymed), rabbit anti-SHP2 (Santa Cruz) or rabbit sera anti-human SAP. Note that SLAM is seen here as a broad band because of its extensive glycosylation[1].

shown). Thus, recruitment of SHP-2 to phosphorylated SLAM was blocked by binding of SAP to the sequence surrounding the most membrane-proximal tyrosine residue, Y281.

As SHP-2 can act as a negative regulator of signal transduction[15], binding of SAP could have a positive effect on co-stimulation by anti-SLAM antibody. To test this, we overexpressed SAP by transient transfection into the Jurkat T cells, together with an interleukin (IL)-2-promoter/luciferase reporter construct and SLAM. Stimulation of these transfected cells with a combination of anti-SLAM and anti-CD3 monoclonal antibodies significantly increased IL-2-promoter/luciferase reporter activation compared with Jurkat cells transfected with SLAM alone (Fig. 9). Anti-SLAM by itself did

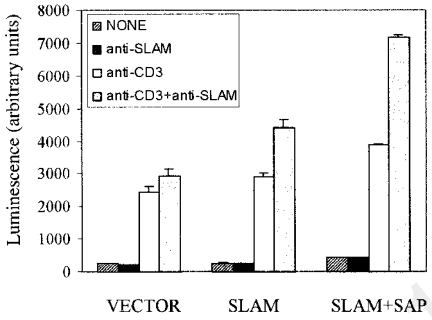

Figure 9 SAP has a positive effect in the SLAM co-stimulatory pathway. Jurkat cells were transfected with SLAM, SAP and SLAM+SAP constructs or vector (pCDNA3), as described in Methods. Luciferase activity was measured after stimulation with the indicated antibodies.

not induce the IL-2-promoter/luciferase reporter signal, and the signal induced by anti-CD3 reagents was weaker than that generated by the two antibodies together. Thus, overexpression of SAP in T cells has a positive effect on the co-stimulatory activity of anti-SLAM.

Discussion

We have identified the gene that is altered in XLP as SAP by using an indirect, functional approach. We have shown that SLAM is the cell-surface protein to which the XLP gene product binds and discovered a new T-cell signal-transduction pathway initiated by the co-receptor molecule SLAM. Binding of the T-cell protein SAP to the cytoplasmic domain of SLAM blocks recruitment of SHP-2. Therefore two modes of SLAM signalling are likely to exist: one in which the inhibitor SAP acts as a negative regulator and another in which SHP-2-dependent signal transduction operates. A switch between these two might occur upon T-cell activation, because expression of the SAP gene drops rapidly after the T-cell antigen receptor is stimulated (C.W., J.S. and C.T., manuscript in preparation). It is possible that SAP is released from SLAM upon activation of T cells to bind to other protein(s). The inhibition by SAP of SH2-domain interactions could be generally extended to molecules other than SLAM. Our results support a model in which SAP controls signal-transduction pathways that are initiated by interactions between SLAM molecules on the interface between T and B cells.

The failure of the immune system to eliminate EBV-infected B cells in XLP appears not to be caused by a B-cell-specific defect, but rather by defective EBV-specific helper-T-cell and cytotoxic-T-cell responses[10–12,16]. As SAP is found predominantly in thymus-derived lymphocytes, SAP mutations should affect SLAM-induced signal-transduction events in T lymphocytes. Many XLP patients have impaired interferon-γ production by helper T cells, indicative of a Th2-like phenotype. Because engagement of SLAM during antigen-specific T-cell stimulation induces IFN-γ production and redirects the Th2 phenotype to a Th1/Th0 phenotype[4], an inadequate response of the T-helper subset in XLP patients could result from impaired functioning of the SLAM/SAP pathway. Alternatively, as reduced EBV-specific cytotoxic-T-cell responses have been reported in XLP patients, EBV-infected B cells may not be 'licensed' to activate T-killer cells by helper T cells that lack SAP[17–19]. In that model, SLAM–SLAM binding in T/B lymphocyte interactions might serve the same purpose as CD40–CD40L interactions in activation of dendritic cells by helper T cells. On the basis of our results, particularly the correlation between the XLP phenotype and loss of binding of the respective SAP mutants to the SAP docking site on SLAM, we conclude that dysfunctional SLAM/SAP-induced signal-transduction pathways are responsible for the ineffective T-cell response in sustaining elimination of EBV-infected B cells.

Uncontrolled B-cell proliferation in XLP patients frequently leads

to non-Hodgkin's lymphomas induced by EBV. This type of non-Hodgkin's lymphoma is often found in AIDS patients, bone-marrow-transplant patients, and individuals who undergo suppressive T-cell treatment. Some of these disease states could be caused by abnormal regulation of the SAP gene and/or of the T-cell subsets that express SAP. □

Methods

Two-hybrid screening. A cDNA encoding the human SLAM cytoplasmic domain was cloned in the pGBT9 vector and used to screen a cDNA library derived from the human T-cell line KT3 in the yeast two-hybrid system[20]. The 5′ end of human SAP was obtained by rapid amplification of closed ends (RACE; Clontech). Mouse SAP cDNA was cloned by screening a mouse thymus cDNA library in vector Zap Express (gift from L. Clayton) using as a probe a cDNA amplified from mouse thymic mRNA by RT-PCR, obtained with specific primers flanking the 5′ and 3′ ends of the expressed-sequence tag (EST) AA255258.

RNA and DNA analysis. For northern blotting, poly(A)$^+$ RNA from various human tissues (Clontech) or from total mRNA from cell lines (Trizol, GIBCO-BRL) was used as described[21] with a radiolabelled human SAP-specific cDNA probe. RT-PCR was done with a GenAmp RT-PCR kit (Perkin Elmer) by using the following primers: forward 5′-GCC TGG CTG CAG TAG CAG CGG CAT CTC CC-3′; reverse, 5′-ATG TAC AAA AGT CCA TTT CAG CTT TGA C-3′. The RT-PCR products were then electrophoresed on 10% polyacrylamide gels at 200 volts for 7 h. Gels were stained with CYBR green (Molecular Probes) for 20 min according to the manufacturer's instructions and visualized and photographed under a UV illuminator. For exon PCR and restriction analysis, each of the four exons of the human SAP gene was amplified by PCR using the following primers: exon 1, forward, 5′-GCC CTA CGT AGT GGG TCC ACA TAC CAA CAG-3′, and reverse 5′-GCA GGA GGC CCA GGG AAT GAA ATC CCC AGC-3′; exon 2, forward, 5′-GGA AAC TGT GGT TGG GCA GAT ACA ATA TGG-3′, and reverse, 5′-GGC TAA ACA GGA CTG GGA CCA AAA TTC TC-3′; exon 3, forward, 5′-GCT CCT CTT GCA GGG AAA TTC AGC CAA CC-3′, and reverse, 5′-GCT ACC TCT CAT TTG ACT TGC TGG CTA CAT C-3′; exon 4, forward, 5′-GAC AGG GAC CTA GGC TCA GGC ATA AAC TGA C-3′, and reverse, 5′-ATG TAC AAA AGT CCA TTT CAG CTT TGA C-3′. The design of these PCR primers was based on the genomic sequence of human SAP (EMBL database). The PCR products were visualized on 2% agarose gels, subcloned into pCR2.1 vector (Invitrogen), and sequenced. For analysis of a point mutation in the region preceding exon 2, exon 2 PCR products were gel-purified (Qiagen) and digested with the restriction enzyme *Mnl*I (New England Biolabs), followed by 10% polyacrylamide gel electrophoresis at 200 volts for 3 h.

Rabbit antisera. SLAM Y2, SLAM Y3, hSAP (CQGTTGIREDPDV) and mSAP (CQAPTGRRDSDI) peptides were coupled to KLH (PIERCE) and used to inject New Zealand rabbits. Rabbit antisera were obtained by standard procedures.

Plasmid construction, transfection and luciferase assays. Human SAP cDNA was cloned in vector pGEX2T (Pharmacia) to generate a GST–SAP construct. Human SAP cDNA and mutant SAP cDNAs, generated by PCR (SAPdel1, SAPdel2, SAPdel3, SAPdel4), were cloned in expression vector pCMV2-Flag (Kodak). CD8–SLAM constructs were generated by PCR as described[22] and cloned in pCDNA3 (Invitrogen). The SLAM construct was generated by subcloning human SLAM cDNA in vector pJFE14-SRα (a gift from DNAX Research Institute). E1-4 cells were stably transfected by electroporation with a cDNA encoding SLAM4 or vector alone. Cells were then selected by growth in medium with 600 mg ml^{-1} neomycin for three weeks, stained with anti-SLAM phycoerythrin-conjugated antibodies (A12), and the positive cells sorted. COS-7 cells were transfected by the DEAE–dextran method[22]; cDNAs coding for SLAM and SAP were transiently transfected with an IL-2 promoter–luciferase construct into Jurkat-Tag cells as described[23]. After 24 h, transfected cells were stimulated with anti-CD3 and/or anti-SLAM monoclonal antibodies as described[1]. Postnuclear lysates were analysed in a Berthold luminometer 8 h later.

Peptide-affinity columns. Whole lysates from untransfected EL-4 cells and from SLAM-transfected EL4-cells were incubated for 3 h with 5 μg GST or GST–SAP protein in the presence of glutathione beads, which were washed three times and separated in SDS–PAGE as described below. Peptides SLAM Y1 (CVEKKSLTI**Y**AQVQK), SLAM pY1 (CVEKKSLTI**pY**AQVQK), SLAM F1 (CVEKKSLTI**F**AQVQK), SLAM Y2 (CTTI**Y**VAATEPVPESVQE) and SLAM Y3 (CTV**Y**ASVTLPES) were synthesized and coupled to the beads (sulpholink-coupling gel; Pierce). Coupled peptides were incubated for 1 h with lysates from mouse thymocytes or from CD8–SLAM3 transiently transfected COS-7 cells, in the presence of different concentrations of soluble peptides as indicated. Beads were washed three times and separated by SDS–PAGE.

Immunoprecipitation. Cell lysates were prepared as described[22], clarified by centrifugation at 14,000*g* for 15 min at 4 °C, and the crude lysate was then precleared using 50 μl protein G–agarose beads (GIBCO-BRL) and 5 μl normal mouse serum or normal rabbit serum for 1 h. Immunoprecipitations were done by using the indicated antibodies and 30 ml protein G–agarose beads for 3 h at 4 °C. Beads were then washed as described[22]. Crude lysates and immunoprecipitates were subjected to SDS–PAGE and transferred onto PVDF filters (Millipore). Filters were blocked for 1 h with 5% skim milk (or 3% bovine serum albumin) and then probed with the indicated antibodies. Bound antibody was revealed using horseradish peroxide-conjugated secondary antibodies using enhanced chemiluminescence (Supersignal; Pierce). For anti-phosphotyrosine blotting, we used a directly conjugated horseradish peroxide antibody cocktail (Pierce).

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1. Cocks, B. G. *et al.* A novel receptor involved in T-cell activation. *Nature* **376,** 260–263 (1995).
2. Aversa, G. *et al.* SLAM and its role in T cell activation and Th cell responses. *Immunol. Cell Biol.* **75,** 202–205 (1997).
3. Aversa, G., Chang, C. J., Carballido, J. M., Cocks, B. G. & de Vries, J. E. Engagement of the signalling lymphocytic activation molecule (SLAM) on activated T cells results in IL-2-independent, cyclosporin A-sensitive T cell proliferation and IFN-gamma production. *J. Immunol.* **158,** 4036–4044 (1997).
4. Carballido, J. M. *et al.* Reversal of human allergic T helper 2 responses by engagement of signaling lymphocytic activation molecule. *J. Immunol.* **159,** 4316–4321 (1997).
5. Isomaki, P. *et al.* Increased expression of signaling lymphocytic activation molecule in patients with rheumatoid arthritis and its role in the regulation of cytokine production in rheumatoid synovium. *J. Immunol.* **159,** 2986–2993 (1997).
6. Punnonen, J. *et al.* Soluble and membrane-bound forms of signaling lymphocytic activation molecule (SLAM) induce proliferation and Ig synthesis by activated human B lymphocytes. *J. Exp. Med.* **185,** 993–1004 (1997).
7. Ferrante, P. *et al.* Cytokine production and surface marker expression in acute and stable multiple sclerosis: altered IL-12 production and augmented signaling lymphocytic activation molecule (SLAM)-expressing lymphocytes in acute multiple sclerosis. *J. Immunol.* **160,** 1514–1521 (1998).
8. Lamartine, J. *et al.* Physical map and cosmid contig encompassing a new interstitial deletion of the X-linked lymphoproliferative syndrome region. *Eur. J. Hum. Genet.* **4,** 342–351 (1996).
9. Porta, G. 4.5-Mb YAC STS contig 50-kb resolution, spanning Xq25 deletions in two patients with lymphoproliferative syndrome. *Genome Res.* **7,** 27–36 (1997).
10. Lanyi, A. *et al.* A yeast artificial chromosome (YAC) contig encompassing the critical region of the X-linked lymphoproliferative disease (XLP) locus. *Genomics* **39,** 55–56 (1997).
11. Purtilo, D. T. Immunopathology of X-linked lymphoproliferative syndrome. *Immunol. Today* **4,** 291–295 (1983).
12. Seemayer, T. A. *et al.* X-linked lymphoproliferative disease: twenty-five years after the discovery. *Res.* **38,** 471–478 (1995).
13. Rousset, F., Souillet, G., Roncarolo, M. G. & Lamelin, J.-P. Studies of EBV-lymphoid cell interactions with the X-linked lymphoproliferative syndrome: normal EBV-specific HLA-restricted cytotoxicity. *Clin. Exp. Immunol.* **63,** 280–289 (1986).
14. Cooper, D. M. & Krawczak, M. *Human Gene Mutations* (Bios Scientific, Oxford, 1993).
15. Marengere, L. E. *et al.* Regulation of T cell receptor signaling by tyrosine phosphatase SYP association with CTLA-4. *Science* **272,** 1170–1173 (1996).
16. Sutkowski, N. *et al.* An Epstein-Barr virus-associated superantigen. *J. Exp. Med.* **184,** 971–800 (1996).
17. Ridge, J. P., Di Rosa, F. & Matzinger, P. A conditioned dendritic cell can be a temporal bridge between a CDR^+ T-helper and a T-killer cell. *Nature* **393,** 474–478 (1998).
18. Schoenberger, S. P., Toes, R. E. M., van der Voort, E. I. H., Offringa, R. & Melief, C. J. M. T-cell help for cytotoxic T lymphocytes is mediated by CD40–CD40L interactions. *Nature* **393,** 480–483 (1998).
19. Bennett, S. R. M. *et al.* Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* **393,** 478–480 (1998).
20. Ramesh, N., Anton, I. M., Hartwig, J. H. & Geha, R. S. WIP, a protein associated with Wiskott-Aldrich syndrome protein, induces actin polymerization and redistribution in lymphoid cells. *Proc. Natl Acad. Sci. USA* **94,** 14671–14676 (1997).
21. Wang, B. *et al.* Over-expression of CD3-ϵ transgenes blocks T lymphocyte development. *Intl. Immunol.* **7,** 435–448 (1995).
22. de Aos, I. *et al.* Tyrosine phosphorylation of the CD3-epsilon subunit of the T cell antigen receptor mediates enhanced association with phosphatidylinositol 3-kinase in Jurkat cells. *J. Biol. Chem.* **40,** 25310–25318 (1997).
23. Martinez-Martinez, S. *et al.* Blockade of T-cell activation by dithiocarbamates involves novel mechanisms of inhibition of nuclear factor of activated T cells. *Mol. Cell. Biol.* **17,** 6437–6447 (1997).

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